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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **309th Meeting** *of the Biochemical Society was held in the School of Biochemistry, Tennis Court Road, Cambridge, on Saturday,* 21 *June* 1952, *when the following papers were read.*

COMMUNICATIONS

The Dialuric Acid Haemolysis Test for Vitamin E Deficiency in Rats. By DOROTHY H. HEARD (*Department of Pathology, University of Cambridge*), T. MOORE and I. M. SHARMAN (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

György & Rose (1949) found that the red blood cells of rats deficient in vitamin E became haemolysed when incubated with a dilute solution of dialuric acid. This abnormality could be corrected by giving the animals adequate doses of α-tocopherol, or even by the addition of tocopherol *in vitro*.

We have confirmed the validity of this test on numerous rats which were restricted to a basal diet deficient in vitamin E, and either dosed or not dosed with α-tocopherol. Although albino and piebald rats show slight differences in their response to vitamin E deficiency (Moore, 1950) the corpuscles of both breeds were liable to haemolysis. Corpuscles from rats deficient in vitamins A or D, but adequate in vitamin E, were not haemolysed. Sometimes haemolysis was observed, however, in corpuscles from rats which had been given diets containing 20% of cod liver oil in conjunction with doses of tocopherol which would otherwise have been adequate. The action of cod liver oil in increasing the requirement for vitamin E is well known. Corpuscles from rats given diets deficient in protein usually resisted haemolysis, but one exception was observed in a rat which had been deficient for over a year, and which was later found to have a fatty liver.

Dam and his colleagues have reported that rats may be protected against most of the effects of vitamin E deficiency by several substances which are not chemically related to the vitamin, including methylene blue. In the haemolysis test Christensen & Dam (1951) found that the dye gave marked but not complete protection. When methylene blue was added to our usual vitamin E-deficient diet, which differs considerably from those used by Dam, there was no protection against haemolysis. Some evidence of a vitamin E synergistic action, however, was seen in the delay of dental depigmentation and of brown discoloration of the uterus.

Examinations in a few human subjects with haemolytic diseases or in calves, under the care of Dr K. L. Blaxter, which had been fed upon excessive amounts of cod liver oil, gave no evidence that the corpuscles were sensitive to haemolysis.

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The Effects of Deoxyribonucleic Acid and Suramin on β-Glucuronidase Activity. By EVELYN E. B. SMITH and G. T. MILLS. (*Biochemistry Department, University of Glasgow*)

Bernfeld & Fishman (1950) have shown that dilution of a purified calf spleen β-glucuronidase preparation causes a decrease in the enzyme activity to protein ratio, and that there is present in boiled enzyme preparations a material which prevents this effect. They postulated that β-glucuronidase requires a coenzyme for full activity, and that this coenzyme dissociates on dilution. They showed that deoxyribonucleic acid (DNA) activates β-glucuronidase at pH 4·5, and also that DNA appears to fulfil the requirements of the postulated coenzyme of calf spleen β-glucuronidase.

In the present work it has been found that dilution of highly purified ox liver and spleen β-glucuronidase causes no decrease in the enzyme activity to protein ratio as found by Bernfeld & Fishman (1950). The activating effect of DNA at pH 4·5 has been confirmed, but it has been found that DNA causes an inhibition of purified ox liver β-glucuronidase at lower pH values. The relation of inhibition

to pH follows an S-shaped curve similar to that obtained for suramin inhibition of β-glucuronidase. The inhibition-pH curves are not identical for these two compounds, the point of 50% inhibition being pH 4·0 for DNA and pH 4·7 for suramin. The effects of DNA on β-glucuronidase are probably explicable on the basis of DNA being a polyacidic compound acting in like manner to suramin.

Some small molecular weight aromatic acids have been studied for their inhibitory effects on β-glucuronidase and these also exhibit an S-shaped pH-inhibition curve.

It is concluded from this work that there is no evidence to substantiate the claim of Bernfeld & Fishman (1950) that DNA is the coenzyme of β-glucuronidase. In addition, it is suggested that the theory of Wills & Wormall (1950) and Wills (1952), that the pH of 50% inhibition of an enzyme by suramin is the isoelectric point of the enzyme, may not apply in the case of β-glucuronidase.

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The Partition of Carotenoids and Vitamin A in the Milk of Cows and Goats throughout Lactation. By R. Chanda. (*The Hannah Dairy Research Institute, Kirkhill, Ayr*)

Using chromatographic and spectrophotometric methods (Chanda, Owen & Cramond, 1951; Chanda & Owen, 1952) the partition of carotenoids and vitamin A in the milk of three cows from the 2nd to the 40th week of lactation and the partition of vitamin A in the milk of two goats from the 2nd to the 30th week of lactation were determined. All the animals were receiving winter rations. In cow milk fat the vitamin A content decreased gradually up to the 30th week. Thereafter, when the yield of milk was decreasing rapidly, both the percentage of fat in the milk and the percentage of vitamin A in the fat showed small but definite increases. A quadratic curve fitted to the data showed a minimum concentration of vitamin A in the fat at 25 weeks *post partum*. In goat milk fat the minimum occurred at 20 weeks.

In goat milk fat, 98% of the vitamin A was present as ester, vitamin A alcohol not being measurable. In cow milk, 5–8 i.u. vitamin A alcohol/100 ml. were always present, and the amount was unaffected by the stage of lactation. Carotenoids, though found in colostrum (Chanda & Owen, 1952), were not present in goat milk at any stage of lactation.

The carotenoid content of cow milk showed no changes as lactation advanced. Between the 2nd and 40th week of lactation, the amount of β-carotene present in cow milk fat ranged from 4·3 to 6·0 μg./g. fat; the mean was 5·1 μg./g. fat, and its coefficient of variation ±5·6%. Of the total carotenoids, β-carotene formed 65–85%, but there was evidence that the percentage was influenced by intake. Variations of the ratio of vitamin A to carotene were almost entirely determined by variations in the vitamin A content, thus presenting the reverse of the conditions found in the blood serum by Goodwin & Wilson (1951).

Goat milk contained 60 i.u. vitamin A/g. fat in the 2nd week of lactation. The corresponding figure for cow milk fat was 49 i.u. (vitamin A + β-carotene × 1·67). This superiority of the goat milk fat over that of the cow persisted throughout lactation, a fact which can perhaps be related to the greater activity of the thyroid gland of the goat which facilitates absorption of carotene and its conversion to vitamin A (Chanda, Clapham, McNaught & Owen, 1951; Chanda & Owen, 1952), and thus makes more of the vitamin available for secretion into the milk.

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The Amino-acid Sequence in the Glycyl Chain of Insulin. By F. SANGER and E. O. P. THOMPSON. (*Biochemical Laboratory, University of Cambridge*)

By oxidation of insulin with performic acid it is possible to split the S—S bridges, and separate two types of polypeptide chain in which the cystine residues have been converted into cysteic acid residues (Sanger, 1949). The amino-acid sequence in the chains with *N*-terminal phenylalanyl residues has previously been reported (Sanger & Tuppy, 1951). The fraction bearing glycyl *N*-terminal residues (fraction *A*) was hydrolysed with 11 N-HCl at 37° for 2–3 days, and the resulting peptides fractionated by paper chromatography after preliminary group separations using adsorption on charcoal and ionophoresis in silica gel or filter paper (Durrum, 1950).

On ionophoresis in 0·2 M-acetic acid peptides containing one cysteic acid residue remained as an approximately neutral band and could be completely separated from other peptides which moved towards the cathode. Those containing more than one cysteic acid residue moved towards the anode and could be identified as discreet bands.

The *N*-terminal residues of the peptides eluted from the chromatograms were determined by the dinitrophenyl (DNP-) method, the reaction being carried out in the presence of trimethylamine which could later be removed *in vacuo*. From the results with the peptides identified in the acid hydrolysate the following sequences were deduced as being present:

Gly . Ileu . Val . Glu . Glu . $CySO_3H$. $CySO_3H$. Ala (*N*-terminal sequence).

Ser . Leu . Tyr . Glu . Leu . Glu . Asp . Tyr . $CySO_3H$.

Ser . Val . $CySO_3H$.

$CySO_3H$. Asp.

When fraction *A* was treated with pepsin, a peptide was split off which contained no aspartic acid or tyrosine. On partial hydrolysis it gave rise to Ser . Val . $CySO_3H$ and Ser . Leu in addition to peptides from the *N*-terminal sequence. From these results it was deduced that the Ser . Val . $CySO_3H$ sequence adjoins the alanine residue in the intact polypeptide chain and the complete amino-acid sequence of the glycyl chain is therefore:

Gly . Ileu . Val . Glu . Glu . CyS . CyS . Ala . Ser . Val . CyS . Ser . Leu . Tyr . Glu . Leu . Glu . Asp . Tyr . CyS . Asp.

A number of other peptides were identified in the peptic hydrolysate and in hydrolysates obtained by the action of chymotrypsin, papain and the mould protease of Crewther & Lennox (1950). These peptides confirmed the above sequence. $CySO_3H$. Asp($—NH_2$) was liberated by the action of chymotrypsin, indicating that the *C*-terminal residue is asparagine. Carboxypeptidase (previously incubated with di*iso*propyl fluorophosphonate to inhibit the small amount of chymotrypsin impurity) liberates free asparagine from the *A* chain but only in small amounts. This is possibly due to inhibition from the adjacent cysteic acid group. Insulin treated with carboxypeptidase yields alanine (from the *B* chains; Lens, 1949) and also an appreciable amount of asparagine. Higher yields have been obtained using acetyl insulin and an approximate estimation by paper chromatography indicated 90 % liberation of the asparagine residues.

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Further Observations on the Amide and Free Carboxyl Groups of Insulin. By A. C. CHIBNALL and M. W. REES. (*Biochemical Laboratory, University of Cambridge*)

When the hydrolysate of reduced insulin (Chibnall & Rees, 1951) was subjected to two-dimensional chromatography (phenol-0·3 % NH_3; butanol-acetic acid), no evidence was found for the presence of γ-hydroxy-α-amino-*n*-butyric acid or its lactone, indicating the absence of aspartyl residues in the insulin molecule. Meanwhile Sanger & Thompson (see preceding communication) had found evidence for the presence of a *C*-terminal asparagine residue.

We have accordingly re-investigated the esterification of this protein by treatment with diazomethane, and, as an alternative procedure, have also used that of Mommaerts & Neurath (1950). On reduction of the ester with $LiBH_4$, we have now obtained the results given in Table 1 which suggest that the insulin molecule (mol.wt. 11700) contains six glutaminyl, eight glutamyl and four asparaginyl residues located in the peptide chains. Two amide

groups must therefore be present as *C*-terminal residues.

Table 1. *Groups per molecule after hydrolysis*

	Insulin	Reduced insulin	Reduced 'oxidized' insulin (Sanger, 1949)
Glutamic acid	14	6	6
Aspartic acid	6	4	4
Total	20	10	10
Amide-NH_3	12	12	12

A *C*-terminal asparagine residue would appear in the reduced insulin hydrolysate as γ-hydroxy-β-amino-*n*-butyric acid or its lactone; these latter products have accordingly been synthesized. Both are retained on a column of Dowex-2 and can thus be separated from β-amino alcohols, which pass through it. Periodate analysis of a reduced insulin hydrolysate fractionated in this way showed the presence of two equivalents of the hydroxyamino-acid and two of alaninol. The β-amino alcohol fraction, nevertheless, gave two equivalents of formaldehyde in excess of those derived from the alaninol. Chromatography and oxidation to the amino-acid showed that this excess was due to the presence of approximately one equivalent of amino-ethanol with a subsidiary amount of leucinol.

When oxidized insulin (Sanger, 1949) was treated in a similar way a corresponding two equivalents excess of formaldehyde were obtained. Neither the glycyl nor phenylalanyl chain gave any such excess, and we confirm the presence in these of a *C*-terminal asparagine and alanine residue respectively (Sanger & Tuppy, 1951; Sanger & Thompson, 1952).

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Determination of the Amino-acid Sequence in Peptides. By J. Leggett Bailey (introduced by A. C. Chibnall). (*Biochemical Laboratory, University of Cambridge*)

The use of lithium aluminium hydride in the preparation of amino alcohols from amino-acid esters was first demonstrated by Karrer, Portmann & Suter (1948). Investigation of the reduction of peptide esters with this reagent has provided evidence of peptide carbonyl reduction. Use of lithium borohydride, as described by Chibnall & Rees (1951), produced intermediate boron complexes not easily split under mild conditions. It has been found that aluminium hydride exhibits a reactivity towards the carbonyl group intermediate between those of the two complex hydrides, and it has now been employed as a selective reducing agent for peptide esters. Reduction carried out at $-40°$ in the medium aluminium chloride-tetrahydrofuran leads to an 85% conversion of the peptide ester to a β-hydroxyalkylamide.

In the degradative procedure under study the β-hydroxyalkylamide of the *N*-*p*-tosyl derivative of a peptide is the starting point. In the presence of such reagents as thionyl chloride or a phosphorus oxyhalide, a rearrangement takes place to give a β-amino ester (cf. Bergmann & Brand, 1923). The latter is then capable of a further treatment with aluminium hydride to yield the free amino alcohol and the residual peptide in a form ready for a further rearrangement:

$$\mathrm{RCONHCHR'CONHCHR''COOEt} \xrightarrow{\mathrm{AlH_3}} \mathrm{RCONHCHR'CONHCHR''CH_2OH} \xrightarrow{\mathrm{POCl_3}}$$

$$\mathrm{RCONHCHR'COOCH_2CHR''NH_2.HCl} \xrightarrow{\mathrm{AlH_3}} \mathrm{RCONHCHR'CH_2OH + HOCH_2CHR''NH_2}.$$

In the case of the hydroxyamide of an *N*-*p*-tosyl amino-acid an 80–85% rearrangement is possible. Preliminary experiments with the hydroxyamide of an *N*-*p*-tosyl dipeptide have indicated slightly less reactivity; a 60% conversion only to the *O*-peptidyl form has so far been achieved. Yields in excess of 90% have been obtained for the liberation of amino alcohols by reductive cleavage of the β-amino ester hydrochlorides employing aluminium hydride. Ion-exchange resins have facilitated the removal of inorganic residues resulting from hydrolysis of the metal hydrides.

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7-Dehydrocholesterol in the Sexual Organs of the Rat. By T. Moore and R. J. Ward. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

The detection in extracts of certain regions of the male rat genital system of prominent absorption bands at 293, 282, 271 and 262 mμ., presumably indicative of the presence of 7-dehydrocholesterol, has already been mentioned (Moore & Ward, 1951). Further experiments have confirmed that this substance is present in the preputial glands, *caput epididymis* and *corpus epididymis*, in descending order of concentration, but not in the testes, *cauda epididymis*, seminal vesicles, coagulating glands and prostates. No selective absorption was seen in the unsaponifiable matter of brain extracts.

The *bulbi vestibuli* of the female, corresponding to the preputial gland in the male, sometimes contained 7-dehydrocholesterol, but none was detected in the ovaries, oviducts and uterus. In wild rats the distribution of 7-dehydrocholesterol was the same as in experimental rats. The preputial glands of two wild rats each contained about 0·4 mg. of 7-dehydrocholesterol per pair, or 2·5 mg./g. of wet gland. In comparison the intestines of normally nourished guinea pigs, according to Glover, Glover & Morton (1952), contain 0·1–0·4 mg./g. of wet tissue.

It is still unknown whether the 7-dehydrocholesterol in the rat genital system has any importance in supplying provitamin D, but theoretically the preputial glands contain enough for conversion into 16 000 i.u. of vitamin D_3. We have detected 7-dehydrocholesterol in the epididymes of rachitic rats, but not in those of sexually immature rats.

Investigations on other animals have so far suggested that the presence of 7-dehydrocholesterol in the male genitals is peculiar to the rat. No selective absorption was seen in extracts of the *caput epididymis* in the human, ram, guinea pig, mouse or rabbit. The preen gland of a duck, examined because of is resemblance to the preputial gland and its supposed role in supplying provitamin D (Hou, 1928) also gave an extract without selective absorption. In a human testis carotene and lycopene were detected, and an extract of the *cauda epididymis* had bands at 278 and 284 mμ. Extracts of the *caput* and *cauda epididymis* of a bull had a band at 260 mμ.

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The Reaction of Haemoglobin with Hydrogen Peroxide. By K. Dalziel and J. R. P. O'Brien. (*Department of Biochemistry (Radcliffe Infirmary), University of Oxford*)

Haemoglobin in dithionite solution, pH 7·0–9·5, is partially changed into choleglobin by reaction with hydrogen peroxide, and spectrokinetic measurements show that an unstable intermediate is formed (Dalziel & O'Brien, 1951). Within a few seconds, the haemoglobin spectrum is completely replaced by a three-banded spectrum, $\lambda_{max.}$ 417, 545 and 580 mμ., which persists with gradually diminishing intensity, and then slowly reverts to a haemoglobin spectrum of reduced intensity with a small additional peak at 630 mμ. Some haemoglobin is lost as products other than choleglobin.

The half-period for the formation of the transient compound is independent of pigment concentration and decreases with increase of peroxide concentration; the life of the compound is prolonged by increased peroxide concentration, and shortened by increased pigment or dithionite concentration, and by catalase; the half-period for the reversion to haemoglobin is independent of pigment concentration and decreases with increase of dithionite concentration. The quantitative spectral absorption and the kinetic data distinguish the compound from oxyhaemoglobin, methaemoglobin and methaemoglobin-hydrogen peroxide.

Carboxyhaemoglobin does not react with peroxide under similar conditions. Prior saturation of the peroxide solution with coal gas also largely inhibits the reaction. But if coal gas is passed into the reaction mixture during the life of the transient compound, much more choleglobin and no less haemoglobin appear in the final products as carboxy-derivatives, suggesting that carbon monoxide inhibits the further degradation of the choleglobin. Carboxyhaemoglobin is formed more slowly than when coal gas is passed into haemoglobin solution. The transient spectrum is gradually replaced by a four-banded spectrum; stable peaks at 420, 539 and 568 mμ., attributed to carboxyhaemoglobin, are fully formed after several minutes, but the fourth peak, at 600 mμ., gradually declines whilst a stable maximum at 628 mμ. builds up, the

intensity at 614 mμ. remaining constant. This change, which takes several hours to go to completion, evidently represents the formation of carboxycholeglobin from a closely related compound.

It is suggested that the transient compound is a haemoglobin-hydrogen peroxide complex, postulated by Lemberg, Legge & Lockwood (1939) as the precursor of choleglobin. The formation of choleglobin is conceived as a stage in the progressive oxidation of the complex by peroxide, presumably by attack at a methine bridge, following the coordination of a peroxide group to the iron atom.

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The Reaction of Methaemoglobin with Hydrogen Peroxide. By K. Dalziel and J. R. P. O'Brien. (*Department of Biochemistry* (*Radcliffe Infirmary*), *University of Oxford*)

The reaction of catalase-free human methaemoglobin with hydrogen peroxide has been studied spectrokinetically, in connexion with the identification of a transient compound formed when haemoglobin reacts with peroxide in dithionite solution (Dalziel & O'Brien, 1951). At pH 6·0–8·5, the initial changes of spectral absorption were consistent with the formation of the unstable complex methaemoglobin-hydrogen peroxide, with an absorption maximum at 545 mμ. and a pronounced shoulder at 575–590 mμ. (Haurowitz, 1935; Keilin & Hartree, 1935, 1951); a Soret band was recorded, $\lambda_{max.}$ 418 mμ. The extinction coefficients ($\epsilon = 1/cd \log I_0/I$, c = m-mole haematin/litre) were $\epsilon_{545} = 10{\cdot}5$ and $\epsilon_{418} = 105$, approximately. With excess peroxide, the reaction was first order with respect to both methaemoglobin and peroxide, in accordance with the equilibrium measurements of Keilin & Hartree (1951) indicating 1 molecule of peroxide per iron atom. The specific reaction rate (m-mole/l.$^{-1}$ sec.$^{-1}$) at 20° was approximately 0·25 at pH 6·0 and 0·025 at pH 8·5.

Slow decomposition of the complex was accompanied by protohaematin destruction, to an extent dependent upon pH and peroxide concentration. At pH 6·0, the spectral absorption of denatured globin carboxyhaemochromogen derivatives prepared at intervals during the reaction showed gradual decline of the carboxyprotohaemochromogen maxima at 539 and 568 mμ., and the progressive development of a maximum at 617 mμ. With an initial methaemoglobin concentration of 0·06 mM with respect to haematin, the extent of this change increased with peroxide concentration up to 0·9 mM, and isosbestic points at 510 and 585 mμ. suggested that other oxidation products were not formed to an appreciable extent. Further increases of initial peroxide concentration resulted in general destruction of specific absorption. The concomitant changes in the spectrum of the reaction mixture were more complex, but indicated that at least one stable pigment was formed in addition to methaemoglobin. The stable products with 0·9 mM-peroxide were green-brown, $\lambda_{max.}$ 585 mμ.; reduction with dithionite after saturation with coal gas gave a residual carboxyhaemoglobin spectrum with an additional peak at 613mμ. The product is evidently not choleglobin, and appears to resemble that obtained by Keilin & Hartree (1951) in the reaction of horse methaemoglobin with peroxide at pH 4·5.

At pH 8·5, no evidence was obtained for the formation of any pigment other than methaemoglobin.

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The Metabolism of Anthranilic Acid in the Rabbit and Rat. By FRANCINE CHARCONNET-HARDING, C. E. DALGLIESH and A. NEUBERGER. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

In investigating the metabolism of tryptophan by B_2-deficient rats (cf. succeeding abstract), large amounts of three substances with a strong purple fluorescence were found in the urines. One of these was identified as anthranilic acid and the others were suspected to be anthranilic acid derivatives. This was confirmed in the present experiments.

Anthranilic acid was fed to normal and B_2-deficient rats (100 mg./day) and normal rabbits (1 g./day) and the metabolites isolated from the urine by the method of Dalgliesh (1952). In all cases examination of the urine by paper chromatography showed essentially the same picture. In butanol-acetic acid (4 : 1 : 5 mixture of Partridge, 1946) there were three main metabolites, α (R_F, 0·56, present in largest amount) and β and γ (R_F's 0·78 and 0·91 present in comparable amounts). In addition smaller amounts of other metabolites appeared, some showing fluorescence typical of anthranilic acid whilst others showed fluorescence compatible with *N*-acylanthranilic acids. These were not examined. No appreciable amounts of hydroxyanthranilic acid derivatives appeared (cf. Bray, Lake, Neal, Thorpe & Wood, 1948).

Substance γ was identified as anthranilic acid and α as its glucuronide, which is already known to be the principal metabolite of anthranilic acid (cf. Williams, 1949) and has been identified under similar circumstances by Mason (1952).

Substance β was suspected to be *o*-aminohippuric acid. This (previously unknown) substance was therefore synthesized. Comparison with β by paper chromatography in a variety of solvents, acidic, basic and neutral, showed the two to behave identically.

Glycine conjugation has been said to be inhibited by the basic *ortho* substituent of anthranilic acid (Muenzen, Cerecedo & Sherwin, 1926; Quick, 1932). It was, however, considered possibly to occur by Bray *et al.* (1948), and the present results bring anthranilic acid into line with other *o*-substituted benzoic acids which have been shown to undergo conjugation with glycine (e.g. Bray, Clowes, Thorpe, White & Wood, 1952).

Preliminary results show that separation of the metabolites can be effected on buffered celite columns.

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Riboflavin and Tryptophan Metabolism in the Rat. By FRANCINE CHARCONNET-HARDING, C. E. DALGLIESH and A. NEUBERGER. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The mammalian conversion of tryptophan to nicotinic acid derivatives (cf. Dalgliesh (1951) for discussion and references) must involve hydroxylation of the benzene ring derived from tryptophan, most probably by conversion of kynurenine or a derivative to 3-hydroxykynurenine or a derivative. It has been suggested (Henderson, Weinstock & Ramasarma, 1951) that riboflavin is involved in this hydroxylation.

Tryptophan was fed to (I) normal, (II) B_2-deficient and (III) B_2- and B_6-deficient rats and the urinary metabolites of tryptophan were examined by methods already described (Dalgliesh, 1952). Both young and adult rats were used.

Urines of B_2-deficient rats contained (besides minor components not examined) (*a*) anthranilic acid, its glucuronide, and *o*-aminohippuric acid which are also found in the urines of normal rats fed anthranilic acid (cf. preceding abstract), (*b*) N^{α}-acetylkynurenine, kynurenine being noted only rarely and in small amount and (*c*) kynurenic and xanthurenic acids. The xanthurenic acid excreted far exceeded that for normal rats, levels of 10 mg./100 mg. administered L-tryptophan being observed, and on adding B_2 to the diet xanthurenic acid excretion immediately returned to normal levels. The urines of normal rats fed L-tryptophan contained negligible amounts of anthranilic acid, kynurenine, hydroxy-

kynurenine or conjugated derivatives. The conversion of tryptophan to anthranilic acid derivatives in B_2-deficient rats (also briefly reported by Mason, 1952) was more marked in the young animals.

Urines from rats deficient in B_2 and B_6 contained no anthranilic acid or its metabolites. This is to be expected as B_6 deficiency inhibits kynureninase, and, moreover, promotes accumulation of kynurenine and related compounds. Both N^{α}-acetylkynurenine and 3-hydroxy-N^{α}-acetylkynurenine appeared, together with kynurenine and sometimes hydroxykynurenine (these two to a greater extent in adult than young rats) and kynurenic and xanthurenic acids.

In all animals essentially the same picture was obtained on replacing supplementary tryptophan by kynurenine, and no conversion of administered kynurenic acid to xanthurenic acid could be detected.

Tryptophan metabolism is thus markedly affected by riboflavin. Riboflavin deficiency does not result in inhibition of hydroxylation (e.g. the high output of xanthurenic acid) but appears to cause diversion, by means not yet clear, of the metabolic pathways to end products of little value.

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Some Properties of Vitamin B_{12}-like Factors from Calf Faeces. 3. Further Biological Properties and Interrelationships. By J. E. Ford, S. K. Kon and J. W. G. Porter. (*National Institute for Research in Dairying, University of Reading*)

We have shown (Ford, Kon & Porter, 1951; Ford & Porter, 1952) that extracts of calf faeces contain four substances having vitamin B_{12} activity for *Escherichia coli*: fraction *A* (now provisionally called cyano-ω-cobalamin), fractions *B* and *C*, and vitamin B_{12} itself.

When *Esch. coli* was grown in a vitamin B_{12}-free basal medium supplemented with fraction *B* (inactive for *Lactobacillus leichmannii*) extracts of the harvested cells contained only fraction *C*, together occasionally with cyano-ω-cobalamin, both markedly active for *Lb. leichmannii*.

Cyano-ω-cobalamin and vitamin B_{12} were recovered largely unchanged after similar passage through *Esch. coli*, although traces of fraction *C* were occasionally detected.

Experiments with chicks depleted of vitamin B_{12} showed that cyano-ω-cobalamin, fraction *B** and vitamin B_{12} injected intramuscularly were partly retained in the livers. Bioautographs of extracts of the livers showed no evidence of interconvertibility of the different substances.

In *in vitro* experiments cyano-ω-cobalamin, fraction *B* or vitamin B_{12} was incubated at 37° with minced livers of depleted chicks in the presence of 0·36% glucose in Krebs bicarbonate saline in equilibrium with 5% CO_2 and 95% O_2. Here again, the different substances appeared unchanged in the extracts after incubation.

Groups of depleted chicks were given cyano-ω-cobalamin (2 or 20 μg./bird), fraction *B* (100 μg.†/bird) or vitamin B_{12} (2 μg./bird) mixed in the basal diet. Vitamin B_{12} was well absorbed and appeared in a concentration of up to 0·4 μg./liver. Cyano-ω-cobalamin was also taken up and appeared as such in the livers, though in much smaller amounts than vitamin B_{12}, even when given at the higher level. Fraction *B* was taken up little, if at all, since it could not be detected in the livers. In some birds, however, the feeding of fraction *B* was accompanied by the appearance of traces of vitamin B_{12} in the livers.

The behaviour of cyano-ω-cobalamin and fractions *B* and *C* suggests that they may be involved in the metabolism of the intestinal flora rather than in that of the host.

We are indebted to our colleague Dr M. E. Coates for the chicks.

* The larger quantities of fraction *B* used in the experiments with chicks were, as before, kindly supplied by Dr E. Lester Smith (cf. Coates, Ford, Harrison, Kon & Porter, 1952).

† As judged by *Esch. coli* assay.

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The Metabolism of *n*-Valeric Acid and Some Branched Chain Acids by Sheep Tissues *in vitro*

By E. F. Annison and R. J. Pennington. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

n-Valeric acid and branched-chain fatty acids together comprise a few per cent of the total volatile acid normally present in the sheep rumen and probably result, at least in part, from deamination of amino-acids by micro-organisms in the rumen (Shazly, 1952). They are probably absorbed from the rumen, as are acetic, propionic and butyric acids (Elsden & Phillipson, 1948), the main fatty acids found in the rumen.

It has recently been shown (Pennington, 1952) that the last-mentioned acids are metabolized by the epithelium of the rumen itself and by other sheep tissues. In the present experiments the fate of *n*-valeric, *iso*butyric, *iso*valeric and racemic α-methylbutyric acids incubated *in vitro* with sheep tissues has been studied. All were found to be metabolized by rumen epithelium; only *n*- and *iso*valeric acids caused an increase in ketone body production over the controls. Generally similar results were obtained with liver slices. Kidney slices metabolized the acids, but with negligible ketone body production with all acids.

*iso*Valeric acid markedly depressed the oxygen uptake of rumen epithelium; Q_{O_2} was increased by the other acids. The mechanism of the respiratory depression is not known; it has been observed also with propionic acid (Pennington, unpublished results).

The uptake of *iso*valeric acid by rumen and liver tissue was much greater in the presence of CO_2 (at the same pH). This observation is in line with the suggestion made by Coon (1950), on the basis of tracer studies, that a CO_2 fixation reaction is involved in the metabolism of *iso*valeric acid by liver tissue.

To investigate whether any of the acids were broken down into lower acids by the tissues, the filtrates, after incubation, were analysed using the gas-liquid partition chromatographic method of James & Martin (1952). Definite evidence of such breakdown was obtained; for example, *n*-valeric acid gave rise to appreciable quantities of acetic and propionic acids.

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Attempts to Render Growth Hormone Non-diabetogenic. By E. Reid (Beit Memorial Research Fellow). (*School of Biochemistry, University of Cambridge*)

The well established finding that diabetes can be induced in cats or dogs by highly purified growth-hormone (GH) preparations, obtained from cattle pituitary glands, does not exclude the possibility that this activity depends on the presence in the preparations of some contaminant. Some evidence against this possibility was obtained by Reid (1952), who subjected such preparations to treatments likely to cause partial inactivation, and in no case found a significant alteration in the 'D/G ratio'—the ratio of diabetogenic activity (assayed in cats) to growth-promoting activity (assayed in rats). In an attempt to obtain further evidence, assays have now been performed on GH preparations treated with thioglycollate, alkali, carboxypeptidase, or oxidized cellulose.

Considerable loss of growth-promoting activity occurred when purified GH preparations were treated with thioglycollate at pH 7·8, or were maintained for 2 days in the cold at pH 13. Growth-promoting activity did not, however, diminish when GH was incubated with carboxypeptidase (Armour) in the presence of di*iso*propyl fluorophosphonate, which served to inhibit the action of any chymotrypsin present in the enzyme preparation (cf. Sanger & Thompson, 1952). Liberation of lysine, serine, alanine, leucine and phenylalanine was demonstrated by partition chromatography. Condliffe & Li (1952) have reported similar findings in experiments with this enzyme.

None of these treatments markedly altered the D/G ratio. The assay data for certain of the treated preparations were suggestive of a rise in the ratio, but in no case were such apparent rises shown to be significant.

Oxidized cellulose has a marked affinity for ACTH but not for GH, and has been used in purifying GH obtained from pig pituitary glands (Raben & Westermeyer, 1951). Pig GH obtained thus, and dissolved at pH 3·5, was tested in dogs by

Raben & Westermeyer (1952), and found to have no diabetogenic activity at doses much higher than the effective dose of beef GH.

Tests now performed on GH preparations treated with oxidized cellulose have not, up to the present, indicated any significant lowering of the D/G ratio. The preparations under test include purified GH (ox), partly purified GH (ox) obtained by the procedure of Raben & Westermeyer (1951), and purified GH (pig) prepared by these authors and kindly provided by them through the courtesy of Prof. E. B. Astwood. This pig preparation, dissolved at pH 10 since it was insoluble at mildly alkaline pH values, has manifested diabetogenic activity in preliminary tests, and is now being quantitatively assayed.

The present experiments have so far given no conclusive evidence against the view previously expressed (Reid, 1952)—that diabetogenic and growth-promoting activity probably depend on the same structures in the same molecule.

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The Direct Determination of Nitrogen in Protein Fractions separated by Electrophoresis in Filter Paper. By A. B. Anderson. (*Biochemical Laboratory, North Middlesex Hospital, London, N.* 18)

The several methods of staining protein fractions after electrophoresis in filter paper, which have been advocated, all have the disadvantage that the dye absorption power of the different fractions varies. The present communication describes a method for the determination of the protein nitrogen directly in the paper. Electrophoresis of serum on strips of Whatman no. 31 paper was carried out in a tank as described by Flynn & de Mayo (1951) using a borate buffer pH 8·7 (Sørensen). At the end of the run the papers were dried at 110° and cut transversely into a series of strips 5 mm. wide. These small strips were individually digested with sulphuric acid selenium dioxide mixture and the nitrogen in the digests determined by nesslerization. Blank determinations on protein free portions of the papers gave a reasonably constant figure.

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Enzymic Oxidation of 5-Hydroxytryptamine in Mammalian and Cephalopod Tissue. By H. Blaschko. (*Department of Pharmacology, University of Oxford*)

Two substances isolated from animal sources are said to be identical with 5-hydroxytryptamine; one is the vasoconstrictor substance serotonin (Rapport, 1949), the other is enteramine (Erspamer & Asero, 1952).

A sample of serotonin creatinine sulphate and one of enteramine picrate have been examined as possible substrates of amine oxidase. The two samples were incubated with extracts from guinea pig tissue and from posterior salivary glands of *Octopus vulgaris*. The rates of oxidation were compared with those of tyramine and tryptamine; it was found that both samples were oxidized by the two tissue preparations at a rate similar to the rate of oxidation of tyramine and of tryptamine. Like tryptamine, 5-hydroxytryptamine forms a dark pigment in the course of the oxidation.

The interest in these observations lies in the high rate of oxidation of 5-hydroxytryptamine; if the amine is proved to be a normal tissue constituent, it may be an important substrate of amine oxidase in the living tissue.

The author is grateful to Prof. J. H. Gaddum and to Dr W. Feldberg for the two samples used in these experiments.

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The Influence of Growth Hormone on the Protein Composition of Rat Muscle. By B. J. HUME (introduced by F. G. YOUNG). (*Biochemical Laboratory, University of Cambridge*)

Bigland & Jehring (1951) showed that the quadriceps muscle from a growth hormone-treated rat was not capable of producing any greater tension than that from a control. Compared gram for gram the treated muscle was actually weaker than the control, suggesting that the extra material laid down under the influence of growth hormone is of a non-contractile nature. A fractionation of the muscle protein has therefore been carried out in order to investigate this problem further.

By extracting with 0·1 M-KCl at 0° followed by 0·1 N-NaOH the muscle protein was divided into three fractions, the sarcoplasmic protein, the myofibrillar protein, and collagen and elastin. Although this procedure does not give a completely clear-cut separation any differences in the gross composition of the muscle protein should be apparent. The results showed no such differences, nor any change in the water content, indicating that the relative proportions of the components of the muscle remained unchanged after growth hormone treatment. Treatment with growth hormone plus testosterone propionate caused a larger increase in weight than with growth hormone alone, but as before the relative composition of the muscle remained unchanged.

In addition, determinations of the 'myofibrillar adenosinetriphosphatase (ATP-ase)', the 'granular ATP-ase' and the 'total ATP-ase' were carried out by a slight modification of the method of Perry (1951, 1952), using homogenates from the quadriceps muscle of growth hormone treated and control rats. A significant decrease was observed in the ATP-ase activity associated with the myofibrils, suggesting that there was a decrease in the relative proportion of myofibrillar protein present. This is in agreement with the findings of Bigland & Jehring that the protein laid down under the influence of growth hormone is not identical with normal muscle protein. The absence of any change in the gross composition of the muscle as determined by the extraction procedure used, is not incompatible with this finding. The part estimated as myosin might be a precursor of myosin or some inert protein of the same nature as myosin but laid down in an unorganized manner. It would then have no ATP-ase activity nor add to the contractile strength of the muscle. Other possible explanations of the findings are also considered.

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Blood Adrenaline Levels During Insulin Hypoglycaemia. By H. WEIL-MALHERBE. (*Research Department, Runwell Hospital, Wickford, Essex*)

Most existing methods for the estimation of adrenaline in blood are unsatisfactory owing to their lack of specificity or sensitivity or both. A method has recently been described (Weil-Malherbe & Bone, 1952) with which, as we believe, fairly reliable values for the sum total of adrenergic amines may be obtained, though it is not possible at present to differentiate between adrenaline and noradrenaline. The term 'blood adrenaline' is here used to signify the total of adrenergic amines estimated by the method without implications as to the identity of the constituents or the composition of the mixture.

The method has been applied to the investigation of adrenaline levels in human venous blood during insulin hypoglycaemia. Many well established observations indicate a state of increased adrenergic discharge during hypoglycaemia; nevertheless, it was found that insulin produces a pronounced fall of the adrenaline concentration in venous blood. The effect is practically instantaneous: after an intravenous injection of insulin the adrenaline concentration curve usually reaches its minimum within 5 min., while the blood sugar may still be within normoglycaemic limits. Thereafter the blood adrenaline level tends to show a gradual rise depending on the dose injected and the response of the subject. If the dose is small (0·1 unit/kg.) and the subject is not abnormally sensitive, the normal adrenaline concentration may be restored after about 20 min., while the blood sugar is still decreasing. Where the response or the dose is greater, the rise of the blood adrenaline is more protracted and the return to the starting level delayed. In any case, a spontaneous upward movement of the blood sugar curve is always preceded by a cor-

responding movement of the blood adrenaline curve. If coma doses of insulin are administered, the adrenaline level remains depressed until the coma is terminated by suitable intervention. In many cases this may be done successfully by the intravenous injection of certain amino-acids (glutamic acid, arginine) or by electric stimulation across the skull. These procedures often lead to a sustained rise of the blood adrenaline up to the starting level or above, followed by a return of consciousness. After the administration of glucose by stomach tube the venous adrenaline level returns to normal within 10–15 min. and often continues to rise for some time afterwards.

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Vitamin A and the Resistance of Rats to Protein Deficiency. By T. Moore, I. M. Sharman and R. J. Ward. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

It has been shown repeatedly that the ability of rats to survive upon diets low in protein is influenced by the intake of vitamin E. In view of the known synergism between vitamins E and A it seemed desirable to find out whether the adequacy of the vitamin A allowance might also affect the resistance to protein deficiency. Eight young male piebald rats were first kept for about 3 months on a diet deficient in vitamin A, but adequate in vitamin E and protein, which was supplemented after the first few weeks with small doses of vitamin A inadequate to allow the deposition of liver reserves. They were then transferred, at body weights of 219–257 g., to a diet containing 10 % of dried yeast as the only source of protein. Four of the animals were dosed with 5000 i.u. of vitamin A acetate daily for 3 days, and subsequently were given 1000 i.u. weekly. With these liberal doses they all increased slightly in weight, and in spite of subsequent declines their mean weight after 10 weeks of dosing was 9 g. above that which had been observed when dosing was started. The other four rats were dosed with only 28 i.u. of vitamin A acetate weekly, which was an allowance calculated to have allowed moderately rapid growth if the basal diet had contained an adequate allowance of protein. All these animals declined in weight. One died after 8 weeks of protein restriction, and after 10 weeks the mean weight of the remaining three rats was 30 g. below the weight at the time when dosing was started. When the same dosage of vitamin A was supplemented by the restoration of an adequate allowance of casein, however, the rats commenced to grow rapidly, with a mean gain of 24 g. in one week. Under the particular conditions of this preliminary experiment, therefore, a liberal allowance of vitamin A enabled the animals at least partially to resist the ill effects of a severe deficiency of protein.

Precursors of Porphyrin in the Urine in Idiopathic Porphyria. By F. K. Herbert. (*Section of Chemical Pathology, Department of Pathology, King's College, Newcastle-upon-Tyne*)

Waldenström & Vahlquist (1939) described the formation of porphobilin and ether-insoluble porphyrins on heating porphobilinogen in acid solution and this was confirmed by Prunty (1945). The increased yield of porphyrin obtained on heating the crude acidified urine, in cases of idiopathic porphyria, has usually been attributed to the formation of porphyrin from porphobilinogen. The recognition of a different type of precursor of coproporphyrin soluble in organic solvents and converted to the porphyrin on oxidation (Raine, 1950; Watson, de Mello, Schwartz, Hawkinson & Bossenmaier, 1951; Eriksen, 1951) suggested the search for an analogous precursor of 'uroporphyrin'. Watson *et al.* (1951) described such a precursor in idiopathic porphyria, separated it from porphobilinogen, and stated that on heating at pH 4·0 porphobilinogen yielded only porphobilin, whereas the new precursor yielded porphyrin.

In the present work, a 'uroporphyrin' precursor, soluble in ethyl acetate and converted to porphyrin on oxidation, has been found in three cases of idiopathic porphyria (in freshly passed urine protected from light). It has been separated from porphobilinogen. Porphobilinogen, as prepared in purified solution by chromatography on alumina, yields both porphobilin and porphyrin on heating in acid solution. The rates of formation of porphyrin and porphobilin, and destruction of porphobilinogen, have been followed in buffered and unbuffered

solutions. In 0·01M-citrate at pH 5·2 the yield of porphobilin is high and porphyrin low. Large amounts of buffer salts suppress porphyrin formation. Maximum porphyrin yields occur in 0·05–0·20N-HCl without buffer salts.

Porphobilinogen solutions in concentrations up to 20 Vahlquist units show no characteristic ultraviolet absorption spectrum. On heating in HCl, the bands of porphyrin and porphobilin develop. In citrate at pH 5·2 the changes on heating are complex and include the transient formation of a weak band at 350–370 mμ. as reported by Brockman & Gray (1951).

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DEMONSTRATIONS

The 'Chromatocoil'—a space-saving device for Paper Chromatograms. By V. Schwarz. (*Research Division, Allen and Hanburys Ltd., Ware, Herts*)

An apparatus has been designed which is small enough to be accommodated in an incubator or refrigerator and thus enables paper chromatograms to be run at any desired temperature.

A wide-mouthed glass-stoppered jar (4·5 cm. diam., 10–11 cm. high) is fitted with a short side arm, also carrying a glass stopper. The jar has two indentations on opposite sides to support a glass rod at a level just below the stopper. Two strips of duralumin, about 5·3 × 1·3 cm., are furnished with 1 cm. slots in such a way as to form four or five prongs (1·5 mm. wide) at both ends of the strips. The two pieces are then fitted together at right angles to each other, forming a cross-shaped holder round which the chromatogram strip can be wound in coil form. The holder carrying the paper strip is suspended from the glass rod and fitted into the jar. A small beaker (5 ml.) contains the stationary phase, while the jar itself holds the moving phase. After equilibration more solvent is added through the side arm until the level just reaches the end of the paper strip projecting towards the bottom. Chromatographic development is thus started and continues until the solvent front reaches the end of the strip when it stops automatically.

Experience has shown that neither the coiling of the strip nor its contact with the prongs of the holder has any untoward effect. The advantages of the apparatus, apart from the great saving in space and hence its ready accommodation in an incubator, are: the short time necessary for equilibration (1–2 hr.); the small volumes of solvents required; the ease with which it can be made in the laboratory.

The use of the apparatus for the chromatography of cortical steroids by the method of Bush (1952) and their location by the arsenomolybdate technique (Schwarz, 1952*a*, *b*) are demonstrated.

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Cytochrome components in Chloroplasts. By H. E. Davenport.

Glycosides and a Glycosidase in the Madder Plant. By A. R. Trim and R. Hill.

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

Studies of sebum. 2. Some constituents of the unsaponifiable matter of human sebum. By R. M. B. MACKENNA V. R. WHEATLEY and A. WORMALL

Spectrophotometric studies on human sebum. By G. N. FESTENSTEIN and R. A. MORTON

The reactivity of radiomimetic compounds. 1. Cross-linking of proteins. By P. ALEXANDER, M. FOX, K. A. STACEY and L. F. SMITH

The effect of fluoride on the succinic oxidase system. By E. C. SLATER and W. D. BONNER, jun.

The action of thrombin on fibrinogen. By L. LORAND and W. R. MIDDLEBROOK

Fibrino-peptide. By L. LORAND

The branched-chain fatty acids of mutton fat. 1. The isolation of (+)-14-methylhexadecanoic acid. By R. P. HANSEN, F. N. SHORLAND and N. J. COOKE

Seasonal variations in fatty acid composition of New Zealand butterfat. By R. P. HANSEN and F. B. SHORLAND

The biosynthesis of protein. 1. Uptake of glycine, serine, valine and lysine by the mammary gland of the rabbit. By P. N. CAMPBELL and T. S. WORK

Addendum—The quantitative estimation in small samples of protein. By S. KROL

Anaerobic and aerobic glycolysis in lactating mammary gland and in nervous tissue. By C. TERNER

Gas-liquid partition chromatography: the separation and micro-estimation of ammonia and the methylamines. By A. T. JAMES, A. J. P. MARTIN and G. HOWARD SMITH

Gas-liquid partition chromatography: the separation of volatile aliphatic amines and of the homologues of pyridine. By A. T. JAMES

Studies in immunochemistry. 11. The isolation and properties of the human blood-group H substance. By E. F. ANNISON and W. T. J. MORGAN

Addendum—Physicochemical examination of blood-group H substance. By R. A. KEKWICK

Citric acid and bone metabolism. By T. F. DIXON and H. R. PERKINS

The determination of glucosamine by alkaline decomposition. By M. V. TRACEY

The metabolism of methyl carbamate. By E. BOYLAND and D. PAPADOPOULOS

Sorbitol antiketogenesis and the dehydrogenation and oxidation of fatty acids. By R. L. BLAKLEY

Sedimentation and diffusion of human albumins. 2. Nephrotic human albumins at a low concentration. By P. A. CHARLWOOD

Studies in the biochemistry of micro-organisms. 89. Metabolic products of *Penicillium multicolor* G.-M. and P. with special reference to sclerotiorine. By J. H. BIRKINSHAW

Phosphates of brain during *in vitro* metabolism: effects of oxygen, glucose, glutamate, glutamine, and calcium and potassium salts. By H. MCILWAIN

Creatine phosphokinase in mammalian brain. By A. NARAYANASWAMI

Effects of insulin and of glycerol *in vitro* on the incorporation of [*carboxy*-^{14}C]acetate into the fatty acids of lactating mammary gland slices with special reference to species differences. By JUDITH H. BALMAIN, S. J. FOLLEY and R. F. GLASCOCK

D-Amino-acid oxidase in the molluscan liver. By H. BLASCHKO and JOYCE HAWKINS

The effect of age on the bulk protein composition of *Chlorella vulgaris*. By L. FOWDEN

The biosynthesis of L-cystine and L-methionine labelled with radioactive sulphur (^{35}S). By R. B. WILLIAMS and R. M. C. DAWSON

The direct isolation of arginine and lysine picrates from gelatin hydrolysates. By W. ROBSON and A. S. M. SELIM

Effect of choline deficiency on the fatty acids of liver and body lipids in the rat. By C. SIVA RAMAN

The effect of light on xanthine oxidase activity. By S. M. SIEGEL and R. L. WEINTRAUB

A method for the preparation of gastrin. By J. E. JORPES, O. JALLING and V. MUTT

The preparation of secretin. By J. E. JORPES and V. MUTT

The free amino-acids of invertebrate nerve. By P. R. LEWIS

A method for the quantitative determination in urinary extracts of C_{21}-17:20-dihydroxy-20-methylsteroids. By R. I. COX

The metabolism of subcutaneously injected [^{15}N] urea in the cat. By H. L. KORNBERG and R. E. DAVIES

The determination of basic groups in collagen and gelatin. By S. C. ELLIS and K. G. A. PANKHURST

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **310th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, The University, Glasgow, on Friday and Saturday, September 19th and 20th, 1952, when the following papers were read:*

COMMUNICATIONS

The Determination of Sodium in Bone. By R. E. Davies, H. L. Kornberg and G. M. Wilson. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry; and Department of Pharmacology and Therapeutics, University of Sheffield*)

The sodium content of dissolved bone cannot be measured directly with a flame photometer. Measurements with this instrument are vitiated by an excess of strong acid, ammonia, calcium, phosphate or oxalate. Furthermore, co-precipitation of sodium occurs with the methods commonly used for the removal of calcium or phosphate; this source of error has been largely overlooked in previous methods for the determination of sodium in bone (Butler & Tuthill, 1931; Harrison, 1937; Forbes & Perley, 1951).

The principle of our method is: ^{24}Na is added to the bone solution, interfering substances are removed by precipitation and sublimation and a correction made, by measurement of the residual radioactivity, for the loss of sodium.

One part of marrow-free bone was dissolved in 2 vol. 70% A.R. HNO_3 and diluted with water to a final concentration of approx. 0·3 g. bone/ml. solution. To a sample (1 ml.) was added 0·01 ml. $^{24}Na_2CO_3$ solution corresponding to approx. 12 000 counts/min., the solution neutralized with ammonia (sp.gr. 0·88) and adjusted to pH 7 with 3N-HCl. Excess (5 ml.) 4% (w/v) ammonium oxalate was added and, after 1 hr., the solution was filtered through Whatman no. 40 filter paper into a 25 ml. Pyrex flask. The filtrate was evaporated to dryness and heated in a muffle furnace at 280° until all ammonium nitrate and oxalate had sublimed (approx. 1 hr.). The charred residue was extracted twice with water and the extract filtered into a measuring tube (vol. of filtrate approx. 7 ml.). Approx. 2 ml. of this solution were diluted to 10 ml. with KCl (20 mg. K^+/100 ml.) and the resultant solution analysed with the flame photometer for the sodium content. The rest of the filtrate was diluted with 0·5 ml. 3M-NaCl (as carrier) and water to 10 ml. The radioactive material was assayed with a liquid-counter tube. Analysis of a solution containing all reagents was carried out to determine the blank correction.

The loss of sodium in the analysis was determined by comparison of the initial and final radioactivities of the solutions; the results obtained by flame photometry were accordingly corrected.

By the use of this technique on pure sodium solutions and on known mixtures of calcium and sodium salts, results correct to $\pm 5\%$ were obtained. The exchange of ^{24}Na with hard glass was negligible. Considerable co-precipitation of sodium with calcium occurred: over 20% of the sodium was still retained even after three washings of the precipitate, showing that the use of labelled sodium was essential to this determination.

The sodium content of human rib (10) was 216–254 (average 239) m-moles/kg. Human femur (6) contained 290–320 (average 300) m-moles/kg., and rabbit femur (16) 267–370 (average 312) m-moles/kg.

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Bile Pigment Formation *in vitro* from Myoglobin. By J. E. Kench and C. Gardikas. (*Departments of Occupational Health and Haematology, University of Manchester*)

This paper describes the coupled oxidation of myoglobin and ascorbic acid investigated as part of a systematic study of the relationship of chemical structure to bile pigment formation (Kench, Gardikas & Wilkinson, 1950; Gardikas & Kench, 1952). Whipple & Robscheit-Robbins (1926) observed increased urobilinuria in dogs following injection of myoglobin, and *in vitro* formation of verdomyoglobin, a choleglobin-like intermediate, has been demonstrated spectroscopically (Kiese &

Kaeske, 1942), but until now the problem of bile pigment production from myoglobin has not been investigated.

In the present work, metmyoglobin was prepared as described by George & Stratmann (1952), the final solution in 3 M-phosphate buffer pH 6·8 being dialysed, and concentrated at 40° to 0·43 mM, measured as pyridine haemochromogen (Rimington, 1942). The solution exhibited the characteristic absorption bands of metmyoglobin at 633·8 and 505·4 mμ., with very weak bands of oxymyoglobin at 582·9 and 547·0 mμ. To 50 ml. 0·43 mM-metmyoglobin solution (≡14 mg. haematin) contained in a Petri dish were added 100 mg. L-ascorbic acid, and the mixture incubated in air at 37° for 2 hr. After 5 min., the band at 633·8 mμ. had almost disappeared, and the bands in the green were much less intense. From 35 min. onwards, increasing absorption was observed in the red region to 620 mμ., and towards the end of incubation the bands of oxymyoglobin became more evident. The solution remained red and clear, in contrast to the cloudy brown appearance of haemoglobin in similar experiments.

Bile pigments were liberated and extracted by the method of Lemberg, Legge & Lockwood (1938), with quantitative determination as fluorescent zinc bilipurpurin (Gardikas, Kench & Wilkinson, 1950). Biliverdin was the sole product, with yields in four experiments of 4·10, 4·42, 5·54 and 4·47, mean 4·70 mg. This yield (36% theoretical) is the highest we have observed from any haematin derivative. A control experiment showed no cholemyoglobin nor free bile pigment admixture in our original metmyoglobin solution. Neither autoxidation during preparation nor incubation at 37° in air, therefore, contributed to biliverdin production. George & Stratmann (1952) were unable to detect either choleglobin or denatured protein during oxidation of myoglobin to metmyoglobin. It is evident that the rapid rate of reaction of myoglobin with O_2 (4·25 times that of haemoglobin) finds a parallel in its increased reactivity with ascorbic acid.

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The Passage of Phosphorus across the Rumen Epithelium of Sheep. By D. PARTHASARATHY, G. A. GARTON and A. T. PHILLIPSON. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

The concentration of inorganic P (25–100 mg. P/100 ml.) in rumen liquor is very high compared to blood (3–9 mg. P/100 ml.). The concentration in venous blood draining the rumen is not, however, higher than in carotid blood or venous blood from the hind legs which indicates that there is no appreciable absorption from the rumen. The present experiments were designed to ascertain whether rumen epithelium was impermeable to inorganic P or whether the permeability was slight.

In acute experiments the rumen was isolated and the ruminal vein and carotid artery exposed. Rumen liquor with or without added phosphate, or approximately isotonic aqueous solutions containing phosphate (3–4 g. P), were introduced into the rumen. Samples of ruminal and carotid blood were taken before and 1 hr. after the introduction of the test solution. There was practically no rise in the blood concentration of inorganic P to indicate absorption whilst the loss in total inorganic P in the rumen was, with one exception, negligible (0·0–0·16 g. P).

In other experiments in which solutions free from phosphate were allowed to remain in the rumen for 1 hr. they were found to contain 0·3–0·9 mg. inorganic P/100 ml., indicating passage from blood to rumen.

Radioactive phosphorus, as ^{32}P-labelled orthophosphate, was added to an approximately isotonic solution containing phosphate or to rumen liquor and the solution introduced into an isolated rumen. The isotope was found in the venous and arterial blood within 15 min., but the percentage absorption was very small (cf. Scarisbrick & Ewer, 1951; Sperber & Hydén, 1952); ^{32}P also appeared in the saliva.

When ^{32}P-labelled phosphate was injected intravenously the isotope appeared in the rumen even against the concentration gradient; the concentra-

tion of the isotope in the liquid in the rumen showed a gradual increase over a period of 1 hr.

These results in general confirm those of Scarisbrick and Ewer (1951) and, in addition, prove that movement of inorganic P occurs in both directions across rumen epithelium.

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The Estimation of the Volatile Fatty Acids formed during the Fermentation of Mixtures of Minced Grass and Water. By A. J. G. Barnett and R. E. B. Duncan. (*Division of Agricultural Biochemistry, Department of Biological Chemistry, University of Aberdeen*)

Determinations of the volatile fatty acids present in silage have been made in the past, using a variety of methods. The technique of Foreman (1920, 1928) as modified by Woodman (1925) gives the total volatile fatty acid content, but it is possible, using formulae due to Dyer (1917) and Wiegner (1926), to estimate acetic and butyric acids separately. Virtanen & Miettinen (1952), using the chromatographic technique of Hiscox & Berridge (1950), has shown that other acids, including propionic, valeric and caproic acids, may occur in silage. The question of the rate of formation of these acids has, however, not been fully investigated because of the difficulty of adequate sampling of ordinary field silage.

The direct estimation of the volatile fatty acids in grass/water mixtures has been studied as a corollary to work in these laboratories on the lactic acid content of silage (Barnett, 1951*a*, *b*, *c*). By means of an apparatus already described (Barnett, 1952) it is possible to sample mixtures of minced grass and water at frequent intervals without upsetting the experimental conditions, and to determine, on the samples so obtained, the amounts of different volatile fatty acids formed, using the gas chromatographic technique of James & Martin (1952).

Preliminary results have shown that, with anaerobic mixtures of similar composition kept at 17°, 37° and 62°, acetic acid is formed in all these, although only to a small extent (0·3 % on the basis of the dry matter present) in the mixtures at 62°. The greatest amounts (2·0 %) are found in the 37° mixtures but in this case butyric acid is also produced in similar quantity.

Lactic acid reaches its maximum concentration (about 8 %, calculated on the dry matter present, under anaerobic conditions) in such mixtures, after 5 days, but the volatile acids are formed much more rapidly. In only one mixture so far studied, has propionic acid been detected.

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The Effect of Thyrotrophin on the Metabolism of Carotenoids and Vitamin A in the Lactating Cow. By R. Chanda, Helen M. Clapham and E. C. Owen. (*The Hannah Dairy Research Institute, Kirkhill, Ayr*)

Blood. The esterified form of vitamin A disappeared from the blood of cows fed on a diet free from carotene and reappeared in amounts which reflected the carotene content of the diet when greenstuff was again allowed. When on refeeding greenstuff 1 ml. (equivalent to 2·5 g. fresh anterior pituitary gland) thyrotrophin ('Ambinon', Organon Laboratories Ltd.) was injected on alternate days the ester form of vitamin A reappeared in the blood in amounts greater than the amount which reappeared in the blood of control cows receiving injections of normal saline. β-Carotene and other carotenoids also

reappeared in amounts reflecting the intake of greenstuff, but their concentration in the blood was, unlike that of vitamin A ester, not further increased by the hormone treatment. On realimentation with greenstuff, there was a small but significant increase in the vitamin A alcohol in the blood, but this increase was unaffected by the hormone. Deprival of carotene for three weeks caused a small decrease in the vitamin A alcohol in the blood. When, however, thyrotrophin treatment was superimposed upon the carotene-free diet the alcohol form of vitamin A in the blood increased markedly. On cessation of thyrotrophin treatment while deprival of carotene still continued a pronounced reverse effect was noticed.

Milk. As in earlier experiments (Chanda & Owen, 1952) a carotene-free diet decreased the carotenoids and carotene in milk fat. On reinstatement of greenstuff, carotenoids and β-carotene were augmented proportionately to the intake of carotene. Treatment with thyrotrophin did not much affect the rate of increase of β-carotene and other carotenoids in the milk on realimentation with greenstuff. This hormone preparation had, however, a marked effect on the rate of increase of vitamin A in the milk and fat on realimentation with greenstuff. This latter increase was all attributable to the ester form of vitamin A and could be related to the simultaneous increase in vitamin A ester in the blood.

As in earlier experiments (Chanda & Owen, 1952) deprival of carotene caused a significant increase in the alcohol form of vitamin A in the milk fat. An important conclusion which arises from the present results is that thyrotrophin resembled thyroxine (Chanda & Owen, 1952) in that it greatly enhanced this increase in the alcohol form of vitamin A. Thyrotrophin was also found to resemble thyroxine in that the increase which it produced in total vitamin A in milk of cows deprived of carotene was mainly in the alcohol form.

In these experiments a further two cows received a Seitz filtrate prepared from beef anterior pituitaries. This preparation was, however, probably inactive, since it was found not to be galactopoietic. It had no effect on the metabolism of carotene.

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The Formation of a Mercapturic Acid from 2:3:5:6-tetrachloronitrobenzene in the Rabbit.

By H. G. Bray, Z. Hybs, Sybil P. James and W. V. Thorpe. (*Department of Physiology, Medical School, University of Birmingham*)

In a previous communication (Bray, Hybs & Thorpe, 1951) the isolation of a mercapturic acid from the urine of rabbits dosed with 2:3:5:6-tetrachloronitrobenzene (TCNB) was described. Evidence has now been obtained that this compound is *N*-acetyl-*S*-(2:3:5:6-tetrachlorophenyl)-L-cysteine. The mechanism of the formation of this mercapturic acid appears to differ from that of other known biosynthetic mercapturic acids since it involves the elimination of a nitro group. In all the aromatic mercapturic acids described in the literature the acetylcysteyl group has replaced a H attached to C and in the known halogenomercapturic acids (formed from monohalogenobenzenes) the acetylcysteyl group has entered in a position *para* to the halogen. 2:3:4:5-Tetrachloronitrobenzene, in which a position *para* to a Cl is available, does not form a mercapturic acid. No mercapturic acid formation was detected when 2:3:5:6-tetrachloroaniline was administered.

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Redox Reactions of Catalase Intermediate Compounds and a new 'Peroxidatic' Role for Catalase. By P. GEORGE. (*Department of Colloid Science, University of Cambridge*)

The titration of compound II of peroxidase with reducing agents (George, 1952*a*) showed it to be one equivalent above the ferric state of the enzyme and thus a compound containing iron with an effective oxidation number +4 and not an enzyme-substrate complex. This leads to a new mechanism for peroxidase action based on one equivalent reduction steps (George 1952*b*):

$$\text{Horse-radish peroxidase} + H_2O_2 \rightarrow \text{compound I} \xrightarrow[\text{red.}]{\text{one equiv.}} \text{compound II} \xrightarrow[\text{red.}]{\text{one equiv.}} \text{HRP.}$$

The reduction of compound I to compound II has subsequently been shown by direct titration (Chance, 1952) thus confirming the new mechanism.

The following experiments, carried out in phosphate buffer solution pH 7·2 in the cuvette of a Unicam spectrophotometer, showed that the spectroscopically similar intermediate compounds formed by catalase show qualitatively the same redox properties. Addition of 16 μM-MeO_2H to 1 μM-catalase provided a mixture of compounds I and II, easily recognized by the spectrum between 380 and 450 mμ., in which compound I slowly changed into compound II. Treating the mixture with potassium ferrocyanide very rapidly gave compound II, which unlike peroxidase compound II was not then reduced to the free enzyme. Catalase compound II is, however, reduced to the free enzyme on addition of $Na_2S_2O_4$, NH_2OH, N_2H_4 or ascorbic acid. Addition of NaN_3 gives azide-ferric-catalase, which suggests that azide also can reduce catalase compound II.

The action of phenols is particularly interesting. Many reduce catalase compound II to the free enzyme and also speed the conversion of compound I into compound II, thus giving a sequence of reactions identical to those of peroxidase. Phenol, hydroquinone, resorcinol, guiacol, *o*-cresol and *p*-cresol react in this way, the steps being most clear with *p*-cresol.

This new reaction path for catalase differs from that postulated by Chance (1949, 1950) for alcohol and nitrite oxidation where apparently direct reduction of compound I to the free enzyme is observed. These two equivalent processes may nevertheless also occur in steps, for further experiments showed nitrite to reduce catalase compound II very rapidly to the free enzyme. With alcohols the first oxidation product formed could react with the compound II produced *in situ*.

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The Respiration of *Nitrosomonas*. By T. HOFMAN and H. LEES. (*Department of Biological Chemistry, University of Aberdeen*)

The respiration of washed suspensions of *Nitrosomonas* has been studied by means of the Warburg technique and the conclusions of Meyerhof (1917) have been reinvestigated. We have confirmed that the O_2 uptake, nitrite formation, and ammonia consumption of the organisms quantitatively conform to the equation:

$$NH_4^+ + 1{\cdot}5O_2 = 2H^+ + NO_2^- + H_2O.$$

Meyerhof noted a sharp drop in respiration rate when the pH of the suspension was decreased below 8·5; in our experiments the nitrite production at pH 6·5 was still 60% of that obtained at the optimal pH, i.e. 8·5. Our results, but not those of Meyerhof, are similar to those obtained when the metabolism of the organisms is studied in whole soils; possible explanations of the discrepancy between our results and those of Meyerhof will be presented. We have confirmed the report of Bömecke (1939) that the organisms have a very small 'rest respiration' in the absence of ammonia. This rest respiration was stimulated by nitrite, also by nitrate, sulphate, or chloride. It will be argued that the stimulation represents the expenditure of energy by some mechanisms concerned in the steady excretion of nitrite diffusing into the cell interior from the medium. The loss of free energy from the cells consequent upon the operation of the 'nitrite excretion mechanism' probably accounts, in part at least, for the fall in free-energy efficiency noted in growing cultures of *Nitrosomonas* (Hofman & Lees, 1952).

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The Intermediary Metabolism of *Nitrosomonas*. By T. Hofman and H. Lees. (*Department of Biological Chemistry, University of Aberdeen*)

Although Kluyver & Donker (1926) advanced the theory that nitrite formation in *Nitrosomonas* followed the path:

ammonia →hydroxylamine →hyponitrite →nitrite,

little experimental evidence for this scheme has so far been forthcoming except for the observation (Lees, 1952*a*) that very low concentrations of hydroxylamine (less than 2 μg. N/ml.) were oxidized to nitrite rather more rapidly than correspondingly low concentrations of ammonia in washed suspensions of *Nitrosomonas*. We can now find that allylthiourea, which is known to inhibit soil nitrification (Lees, 1946) and nitrite formation in washed suspensions of *Nitrosomonas* (Lees, 1952*b*), is a selective poison for the first stage of the Kluyver & Donker scheme. Washed suspensions of *Nitrosomonas*, studied in the Warburg apparatus, showed no O_2 uptake and no nitrite formation when supplied with ammonia in the presence of allylthiourea (10^{-5}M); O_2 uptake and nitrite formation were normal in the presence of this concentration of allylthiourea when the suspensions were supplied with hydroxylamine in place of ammonia. By similar techniques it has been found that hydrazine inhibits hydroxylamine oxidation. Suspensions treated with hydrazine (5×10^{-3}M) accumulated hydroxylamine when supplied with ammonia; the hydroxylamine was detected by the method of Csaky (1949). These results are, to the best of our knowledge, the first demonstration that hydroxylamine is an intermediate in ammonia oxidation by *Nitrosomonas*.

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Secretory Activity of Mitochondria. By W. Bartley and R. E. Davies. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield* 10)

Mitochondria from sheep kidney cortex homogenates are able to do secretory work by actively maintaining concentration differences of both inorganic and organic ions between themselves and the external fluid. This ability is labile, depends on active metabolism, and is reduced or abolished by cooling.

Sheep kidney cortex (50 g.) was homogenized for 2 min. in a Waring Blendor in 200 ml. 0·25M-sucrose containing 2 ml. 1M-NaOH. This and subsequent procedures were carried out at 0°. The resulting material was spun at 700 *g* in a refrigerated centrifuge for 15 min. and the precipitate of nuclei and cell debris discarded. To 40 ml. of the supernatant solution were added 4 ml. 1·5M-KCl and the mixture spun at 4200 *g* for 15 min. The precipitated mitochondria were then washed twice with 0·12M-KCl. Some of the effects were also obtained with 'cyclophorase' preparations (Green, Loomis & Auerbach, 1948) which contained nuclei also. The particles (15 mg. dry wt./ml. solution) were incubated aerobically at 20° in the presence of 10^{-3}M-ATP, 10^{-3}M-Mg^{++}, approx. 10^{-2}M-phosphate (pH 7·4) and substrates such as pyruvate, α-ketoglutarate, fumarate, oxaloacetate.

It was essential to maintain active metabolism at 20° during the subsequent separation of the mitochondria because the ionic concentration differences were reduced or abolished if the preparations were centrifuged at temperatures near 0°.

In spun-down material containing about 40% extra-particulate fluid and approximately 20% solid in the particles the following ratios internal concentration/external concentration have been observed for the spun-down material relative to the supernatant fluid: H^+, 2·5; Na^+, 26; K^+, 2·0; Mg^{++}, 4·5; orthophosphate, 6·0; ATP, 0·7; pyruvate, 0·8; fumarate, 8·0; oxaloacetate, 0·1. The absolute value of these ratios depended critically on the experimental conditions and varied from preparation to preparation; sometimes no evidence was obtained for secretory activity. Experiments with $^{42}K^+$ showed that the turnover of the potassium of the mitochondria was immeasurably high using present techniques. Even if the $^{42}K^+$ was added to the mitochondrial suspension in centrifuge tubes which were spun at once, the specific radioactivities of the potassium ions inside and outside the mitochondria were identical. Since the mitochondria were also able to cause added inorganic $^{32}PO_4^{3-}$ to reach radio-

active equilibrium with external ATP in about 5 min. this rapid turnover of K^+ is expected under these experimental conditions on the basis of the theory of ion transport put forward by Davies & Ogston (1950).

These results demonstrate that isolated subcellular particles are able to do secretory work on simple ions. It may be that much of the secretory activity of some cells depends on the mitochondria, and it follows that there are probably ionic concentration gradients within cells.

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Cortisone, Growth Hormone, and Insulin Action on Embryonic Tissue Growing *in vitro*. By I. Leslie. (*Department of Biochemistry, The University of Glasgow*)

Chick heart explants (48 per tube) from 13-day embryos were grown in roller tubes, and changes in cell number and composition were based on the content of deoxyribonucleic acid phosphorus (DNAP) as previously described (Davidson & Leslie, 1951). By the use of a new type of roller tube, it has been possible to confirm the relative constancy of the average amount of DNAP per nucleus in explants growing for 96 and 144 hr.

The earlier work (Leslie & Davidson, 1951) on the action of insulin has been extended to the study of the action of cortisone, pituitary growth hormone (kindly supplied by Dr E. Reid of Cambridge) and insulin, singly and together, on explants growing over 144 hr. in embryo extract and serum (EES).

Cortisone (15 μg./ml. EES) did not affect the total synthesis of ribonucleic acid phosphorus (RNAP) and DNAP, and this evidence that cell multiplication is unimpaired at this dosage is in agreement with the observations of Cornman (1951) and Barski & de Brion (1952).

With growth hormone (0·1 or 0·5 mg./ml. EES) the average cell number at 144 hr. was about 25% lower than in the controls, but there was no significant change in cell composition.

Cortisone and growth hormone in combination increased the final cell number by as much as 75% above the control level with corresponding increases in lipid phosphorus (LP) and RNAP. In terms of amounts per cell, the LP and RNAP were closely similar to the normal values.

Insulin (2 units/ml. EES) and cortisone, together, produced an increase in total synthesis of LP, RNAP and DNAP, and both LP and RNAP were increased significantly in amount per cell, whereas with insulin alone only the RNAP was increased significantly (cf. Altman, Miller & Bly, 1951).

Insulin and growth hormone in combination gave results similar to those obtained with insulin, namely increased total synthesis of LP, RNAP and DNAP.

All three hormones administered together in the dosage given above caused a marked stimulation of growth, the total synthesis of all fractions (except ASP) being increased by 50–100%. The average amounts of LP and RNAP per cell were raised above the normal levels.

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Effect of pH on Growth of *Aerobacter aerogenes* and *Escherichia coli* in Synthetic Media. By S. Dagley, E. A. Dawes and Shelagh M. Foster. (*Departments of Biochemistry, Universities of Leeds and Glasgow*)

Growth of *Aerobacter aerogenes* and *Escherichia coli* in glucose-ammonium salt medium was followed and pH values of filtrates determined within 1 hr. of growth cessation. With excess substrates, decrease in initial pH from 7·0 lowered stationary populations for both aerated and unaerated cultures, more rapidly for *Esch. coli* than for *A. aerogenes*. Aeration of a medium at any particular pH gave increased total crop and, for cultures inoculated at pH < 7·0, the pH fell to lower values in aerated than in unaerated media. When a culture of *A. aerogenes* which had reached its (unaerated) stationary phase was then aerated, growth was resumed; if pH at inoculation was 5·0–6·0, pH continued to fall on

aeration and if 6·1–7·0, pH rose slightly. These results are not compatible with the suggestion of Ravin (1952) that resumption of growth on aeration of (unaerated) stationary phase cultures may be attributed solely to creation of more favourable pH by oxidation of accumulated organic acids. For a citrate medium, stationary populations of *A. aerogenes* decreased when initial pH was > or < 7·0 in unaerated cultures. Changes in pH during growth were small and for initial values 5·0–6·6 the pH on growth cessation was less than 7·0. Considerably higher bacterial populations resulted in aerated media. When a culture in its (unaerated) stationary phase was aerated cell division recommenced, and so did the pH movement towards alkalinity; citric acid concentration also decreased.

In unaerated glucose cultures growth ceased at pH values which permit growth in fresh media, suggesting that compounds are formed which become increasingly toxic as pH falls. Gale and Epps (1942) showed that 2 % sodium formate stops growth of *Esch. coli* at pH values < 6·2. We determined formic acid in filtrates from fully grown unaerated glucose cultures of both organisms and showed that this amount of formate had little effect at pH 7·0 but abolished growth of *A. aerogenes* in fresh medium at pH 5·0 and of *Esch. coli* at pH 5·25. This work indicates that in systems where oxygen is the ultimate hydrogen acceptor growth is possible under conditions prohibiting anaerobic growth. The effect of aeration is not simply to create a shift to more favourable conditions.

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The Incorporation of ^{32}P and ^{14}C into the Nucleic Acids of Liver Cell Fractions. By R. M. S. Smellie and W. M. McIndoe. (*Department of Biochemistry, The University of Glasgow*)

The uptake of ^{32}P by the ribonucleotides derived from the ribonucleic acid (RNA) of rat and rabbit liver nuclei and cytoplasmic fractions has been studied at various intervals after administration of the isotope. Separation of the nucleotides was by the ionophoretic procedure of Davidson & Smellie (1952*a*, *b*).

It was found in agreement with several authors (Marshak, 1948; Marshak & Calvert, 1949; Barnum & Huseby, 1950; Jeener & Szafarz, 1950; Davidson, McIndoe & Smellie, 1951) that incorporation took place most rapidly and reached a higher level in the nucleotides from the nuclear RNA than from the RNA of any of the cytoplasmic fractions. In the cytoplasm, the 'cell sap' took up the isotope initially at a slightly greater rate than either of the particulate fractions (Jeener & Szafarz, 1950; Barnum & Huseby, 1950; Davidson *et al.* 1951).

At all but the shortest time invervals, no apparent difference in specific activity was observed between the mitochondria and microsome fractions (Barnum & Huseby, 1950), but at 2 hr. as previously reported (Davidson *et al.* 1951) the microsome fraction exhibited a slightly lower specific activity than did the mitochondria.

Of the nucleotides, adenylic acid had in general a slightly higher activity and guanylic acid a slightly lower activity than either of the pyrimidine nucleotides. The previously reported high value for uridylic acid (Davidson *et al.* 1951) has been found to be due to the presence of accompanying substances of high activity (Davidson and Smellie, 1952*b*).

When [^{14}C] formate was administered to rats 4 hr. before sacrifice, the results obtained confirmed the general pattern found with ^{32}P.

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Biological Values and Digestibilities of some Grasses, and Protein Preparations from Young and Mature Species, by the Thomas-Mitchell Method, using Rats. By M. DAVIES and W. C. EVANS (*Department of Agricultural Chemistry, Bangor*) and W. H. PARR (*now at Veterinary Laboratory, Weybridge*)

Chibnall (1939), Lugg (1946) and collaborators found that the amino-acid composition of leaf proteins were very similar; species, age of leaf, manurial treatment, and climate seemed to have little effect upon leaf-protein composition. Armstrong (1951) and Smith & Agiza (1951) claim that species differences occur, and that stage of maturity, conditions of growth, etc. have their effect on amino-acid analyses results of the 'whole-leaf protein'. Several workers have suggested differences in protein quality amongst herbage species, from nutritional experiments. Use of the rat for biological values of pasture plants is limited to the young stage (high protein) only; mature plants have too low a ratio protein/cellulose and lignin, for them to be used directly. In the present work we have attempted to overcome this difficulty by the preparation of a 'whole-leaf protein' fraction from young and mature plants, by 'defiberization' of fresh pure species. Rats (Wistar, weight 50–60 g.) were used, employing the metabolism cages of Henry, Kon & Watson (1937) in the Thomas-Mitchell method, the diet being adjusted to a 10% protein level.

From the results obtained, we tentatively conclude that, (*a*) at the young stage of growth there appears to be little difference in the biological value (B.V.) of the proteins obtained from different species, (*b*) increase in age of the plant is accompanied by a decrease in both B.V. and digestibility, of the leaf proteins.

A cocksfoot (S.143) leaf extract was separated into 'cytoplasmic' and 'chloroplastic-rich' protein fractions by the method of Chibnall (1939). The B.V. of the cytoplasmic protein (crude protein 71·4%) fraction was 60·7, digestibility (D.) 74·8; the chloroplastic protein (crude protein 41·6%) fraction had B.V. 46, and D. 61·2.

It appears that these two main fractions of the 'whole-leaf protein' possess appreciably different biological values.

Results obtained with leguminous pasture species etc. will be discussed, and also apparent discrepancies between chemical and biological data.

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Pathways in the Metabolism of the Monohydroxybenzoic Acids by Soil Bacteria. By N. WALKER (*Soil Microbiology Section, Rothamsted Experimental Station, Harpenden*) and W. C. EVANS (*Department of Agricultural Chemistry, Bangor*)

It had previously been established by Evans (1947) that *p*-hydroxybenzoic acid is metabolized *via* 3:4-dihydroxybenzoic acid by Vibrio O/1. Sleeper & Stanier (1950), working with *Pseudomonas fluorescens* organisms showed that the adaptive pattern of these microbes when grown on *p*-hydroxybenzoate was in accordance with this view. Little attention had been given to the other monohydroxybenzoic acids, partly because Vibrio O/1 failed to grow in a salicylate-mineral salt medium, and growth was poor when *m*-hydroxybenzoic acid was used as substrate.

We have now found that many soil *Ps. fluorescens* strains will grow in a simple mineral salt medium with ease, utilizing either salicylic acid or *m*-hydroxybenzoic acid as sole source of organic carbon. Salicylate grown cells are simultaneously adapted to catechol, but to none of the possible dihydroxybenzoic acids or phenol. These cells also contain, preformed, the enzyme system which converts *cis-cis*-muconic acid to β-ketoadipic acid. A strain of *Ps. fluorescens* growing in a *m*-hydroxybenzoate-mineral salt medium is simultaneously adapted to gentisic acid (2:5-dihydroxybenzoic acid) only. This acid has been identified in the growing culture chromatographically, and subsequently isolated chemically. Cells grown on *m*-hydroxybenzoate when incubated with gentisate, produce a substance

behaving like pyruvic acid in the Friedmann & Haugen (1943) method for the estimation of this acid. A blue colour in the Rothera reaction is also produced. Work continues on the identification of the immediate product of ring cleavage of gentisic acid.

The initial stages in the bacterial metabolism of the three monohydroxybenzoic acids are therefore separate and distinct:

(1) *o*-hydroxybenzoic acid → catechol → subsequent pathway known.*

(2) *m*-hydroxybenzoic acid → 2:5-dihydroxybenzoic acid → ?

(3) *p*-hydroxybenzoic acid → 3:4-dihydroxybenzoic acid → pathway known.*

* See Evans, Smith, Linstead & Elvidge.

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The Influence of Steroids on Citrate Metabolism. By J. K. Grant and W. Taylor. (*Biochemistry Department, University of Edinburgh*)

Components of the tricarboxylic acid cycle have been implicated in the *in vitro* metabolism of steroid hormones. Thus Hayano & Dorfman (1952) reported that fumarate, malate or succinate accelerated C-11 hydroxylation of corticosteroids by adrenal homogenates. Sweat & Samuels (1948) observed that citrate increased the disappearance of the $\alpha\beta$ unsaturated ketonic grouping of testosterone on incubation with liver. The intervention of steroid hormones in the operation of the tricarboxylic acid cycle was suggested by observations of Du Bois, Cochran & Zerwick (1951) that citrate accumulates in livers of fluoroacetate-poisoned female rats but not in females receiving testosterone nor in males.

The influence of various steroids on the *in vitro* metabolism of citrate has now been studied.

Using mitochondria from liver of ovariectomized rats, and with pyruvate and malate as substrates (Kennedy & Lehninger, 1949), testosterone and androsterone (600 μM) caused 60 % (9) and 50 % (2) inhibition of citrate synthesis. The following C_{21} steroids tested at concentrations of 130 and 260 μM caused inhibition of citrate synthesis: progesterone 79 % (15), 86 % (7); 17-hydroxy-progesterone, 1·5 % (3), 16 % (2); 17-hydroxy-deoxycorticosterone, 0 % (5), 33 % (6); deoxycorticosterone, 39 % (6), 76 % (3); cortisone and corticosterone (130 μM) appear to increase citrate synthesis 24 % (4) and 39 % (6). Co I in concentrations up to 0·01 M had no effect on the inhibition obtained with 130 μM progesterone. (Figures in parentheses are the numbers of experiments.) Citrate was determined by an adaptation of the method of Weil-Malherbe & Bone (1949). Similar results were obtained with oxaloacetate as substrate. In a typical experiment the oxygen uptake of the system was reduced 88 % by 130 μM progesterone. In four experiments 9 to 21 % of added progesterone (measured by absorption at 240 mμ. following partition chromatography) disappeared while citrate synthesis was inhibited 80 %. In a fifth experiment no change in progesterone was observed although citrate synthesis was strongly inhibited. These results confirm in part and extend recent observations of Cochran & Du Bois (1952) on the inhibitory action of steroid hormones on citrate synthesis. The mechanism of inhibition is under investigation.

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Changes in the Nucleic Acid Fractions during Tryptophan-tryptophanase Adaptation. By A. A. HAKIM and F. C. HAPPOLD. (*Department of Biochemistry, School of Medicine, Leeds*)

Escherichia coli was grown on a basal medium containing 2% sodium succinate, 1% ammonium sulphate, 1% potassium sulphate, 0·5% sodium sulphate and Mg^{++}, with two different phosphate concentrations (that containing 0·9% NaH_2PO_2 is called the normal phosphate medium whilst a concentration of 0·09% NaH_2PO_4 is referred to as the phosphate-deficient medium).

Suspensions of cells harvested during the logarithmic phase were centrifuged and washed with distilled water. The resulting cells were resuspended in 0·1% tryptophan 0·0133M-phosphate buffer (pH 7·8) solution for adaptation. This simple adaptation was modified in three further portions by the addition of (1) pyridoxal phosphate (PP), (2) ATP and (3) of pyridoxal phosphate + ATP jointly to the normal adaptation mixture. All four mixtures were incubated at 37°. Portions from each sample were taken simultaneously after 0, 10, 60, 120, 180, and 240 min.; the activity of each portion is determined as well as its ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) content. The results showed:

(1) Adaptation as determined by tryptophanase activity is much faster in the phosphate-deficient medium than in the normal medium.

(2) Adaptation is accompanied by a rise in the DNA of both the cellular and supernatant fractions; this is particularly marked in the supernatant (80-fold increase). Changes in the RNA fractions are much less marked.

(3) The presence of exogenous pyridoxal phosphate during the cellular adaptations shows no effect on the adaptation rate nor on the DNA and RNA concentrations.

(4) The joint presence of ATP and PP either retards or inhibits enzyme adaptation.

Ultraviolet spectrophotometer readings show an increase in total nucleic acids during the adaptation process.

A Manometric Method for the Study of Arylsulphatase. By J. M. LOWENSTEIN and L. YOUNG. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

The methods which have been described hitherto for the measurement of the activity of arylsulphatase have depended on the gravimetric, colorimetric or spectrophotometric estimation of one of the products of the hydrolysis of the substrate (cf. Dodgson & Spencer, 1952; Robinson, Smith, Spencer & Williams, 1952; Young, Morrison & Whitehead, 1952).

The hydrolysis of one molecule of a monoarylsulphate, such as potassium phenylsulphate, is accompanied by the production of one acidic hydrogen atom. If arylsulphatase acts on one of its substrates in a $NaHCO_3$-H_2CO_3 buffer, an evolution of CO_2 occurs as the reaction proceeds and this may be measured by means of the Warburg technique. In the absence of CO_2 retention by other buffers such as proteins, one molecule of CO_2 gas released from the buffer corresponds to the hydrolysis of one molecule of monoarylsulphate.

In the present investigation the customary method of measuring CO_2 production by the Warburg technique was employed. The main compartment of the Warburg flask contained 3 ml. of a $NaHCO_3$ solution of the enzyme, and 1 ml. of a $NaHCO_3$ solution of the substrate was placed in the side arm of the flask. Retention of CO_2 was found to be negligible when an arylsulphatase preparation from *Aspergillus oryzae* (Mylase L-1, Wallerstein Laboratories, New York) was used. Experiments in which potassium 1-naphthylsulphate and potassium 4-nitrophenylsulphate were used as substrates showed that the amount of hydrolysis found by manometric measurement agreed with that obtained by colorimetric determination of the phenolic compound liberated in the reaction.

The manometric method has been used for the study of the enzymic hydrolysis of the sulphuric acid esters of phenol, *o*-cresol, 2:6-xylenol, 1-naphthol, 2-naphthol, 4-nitrophenol and oestrone, and the results obtained will be discussed.

The authors wish to thank the Wallerstein Laboratories, New York, for a generous gift of Mylase L-1.

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The Isolation and some Chemical Properties of Viridicatin, a Metabolic Product of *Penicillium viridicatum* Westling. By K. G. Cunningham and G. G. Freeman. (*Research Department, Imperial Chemical Industries Ltd., Nobel Division, Stevenston, Ayrshire*)

Carbon balances of three strains of *Penicillium viridicatum* Westling were reported by Birkinshaw, Charles, Hetherington & Raistrick (1931), who found large amounts of 'carbon unaccounted for' in cultures in Czapek-Dox medium. No other biochemical studies on this species have been reported. Viridicatin was first isolated in 1948 from freshly harvested dried mycelium from a strain of *P. viridicatum* of unknown origin (our collection number F 22) grown on Czapek-Dox medium. Chloroform extraction of the air-dried, ground mycelium gave a colourless crystalline product (yield of crude product, 4·1 %), for which the name viridicatin is proposed. Subsequent ethanolic extraction of the mycelium gave mannitol in yields of 3–4 %. When chemical work on the substance was resumed in 1951, freshly harvested batches of mycelium from the same strain gave only 0·1 % of product, identical with that previously isolated. Enrichment of the medium with thiamine hydrochloride, calcium D-pantothenate, inositol and biotin gave vigorous growth of the mould but no increase in yield of viridicatin. The same results were obtained with beer wort medium and a range of media containing 2–5 g./100 ml. glucose and 0·25–1·0 g./100 ml. of 'Pronutrin' (an enzymic casein hydrolysate). Furthermore, a culture of Raper & Thom's (1949) reference strain (N.R.R.L. 963) obtained from the Commonwealth Mycological Institute and another strain of *P. viridicatum* from the same source (I.M.I. 49162) both failed to yield viridicatin on various media.

Viridicatin crystallized from ethanol as lustrous needles, m.p. 269°. It is sparingly soluble in cold organic solvents and in dilute mineral acids, but is much more soluble in boiling, concentrated hydrochloric acid, from which it separated unchanged on cooling. No optical rotation could be detected in glacial acid solutions of viridicatin. The compound does not contain carboxyl, methoxyl, carbon-methyl or nitrogen-methyl groups. Analysis of the compound and of seven derivatives, which have been prepared, support the molecular formula, $C_{15}H_{11}O_2N$ for viridicatin. The available evidence suggests a polycyclic aromatic structure with a phenolic hydroxyl group and probably a cyclic amide. Viridicatin showed slight *in vitro* antibiotic activity against *Mycobacterium tuberculosis*.

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Action of Sheep Liver Homogenates on Quinine, Quinidine, Cinchonine and Cinchonidine. By G. P. Fraser and W. O. Kermack. (*Department of Biological Chemistry, Aberdeen University*)

Kelsey, Geiling, Oldham & Dearborn (1944) reported that when quinine was incubated with rabbit liver homogenate, a compound was formed which Mead & Koepfli (1944) proved to be 2′-hydroxyquinine. Brodie, Baer & Craig (1951), in experiments on humans, found that after administration of quinine, quinidine, cinchonine and cinchonidine by mouth, various oxygenated derivatives appeared in the urine including compounds believed to be the 2′-hydroxy derivatives of the alkaloids.

In order to study the action of sheep liver on these alkaloids, we have prepared homogenates in Ringer-Locke solution containing approximately 250 g. of liver and 2·5 g. of the alkaloid hydrochloride per litre. Glucose and sodium bicarbonate were added in the proportion of 1 g. per litre to the final solution. The incubation was carried out at 37° for 12 hr., the mixture being stirred and oxygenated throughout by a vigorous air stream.

From quinine two metabolites were obtained; the first 2′-hydroxyquinine, m.p. 251°, identical with that isolated by Kelsey *et al.* (1944) and Brodie *et al.* (1951); the second, m.p. 214–215°, which from its ultraviolet absorption curve has an intact quinoline nucleus, is apparently modified in the quinuclidine part of the molecule. Similarly, from quinidine was obtained 2′-hydroxyquinidine, m.p. 235–260° (cf. Brodie *et al.* 1951) and a second product, m.p. 244–245°, modified in the quinuclidine system. Cinchonidine gave two metabolites, hydroxylated in the quinoline ring, the first, m.p. 229°, having two additional oxygen atoms and corresponding to a substance obtained by Brodie *et al.* (1951). The second compound appears to be identical with one isolated by these authors; this they believed to be 2′-hydroxycinchonidine but the compound isolated by us appears to contain two more oxygen atoms than cinchonidine. From cinchonine, which was least

metabolized, only one compound, m.p. 270–273°, was isolated in small amount; this is apparently identical with the 2′-hydroxycinchonine of Brodie *et al.* (1951).

Kelsey & Oldham (1943) claimed that sheep liver homogenates have little action on quinine but it appears from the present work that although considerably less active than the highly active rabbit liver, sheep liver under the conditions employed is capable of oxidizing the cinchona alkaloids, particularly cinchonidine. The products of oxidation are closely similar to those found in human urine after the administration of these drugs.

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The Excretion of Pteroylglutamic Acid and Citrovorum Factor in Health and Disease. By R. H. Girdwood. (*Department of Medicine, University of Edinburgh*)

It has been shown by Swendseid, Bird, Brown & Bethell (1947) that when 3–5 mg. doses of pteroylglutamic acid are given by mouth, the urinary excretion is less in pernicious anaemia patients than in normals. This is also true when 5 mg. of pteroylglutamic acid are given by injection (Girdwood, 1951). The excretion of an oral test dose is diminished in leukaemia (Swendseid, Swanson, Meyers & Bethell, 1952).

When 5 mg. of pteroylglutamic acid were given intramuscularly, the mean urinary excretion in eight normal persons was 2·51 mg. (range 1·73–3·56 mg.). In eight persons with iron-deficiency anaemia the output was less than that of the controls in four cases (0·6, 0·75, 0·79 and 0·999 mg. respectively). These four were all found to be suffering from inoperable carcinoma. The excretion of the test dose was also impaired in three patients with pyrexial lymphadenoma, but not in six patients with extensive pulmonary tuberculosis. On the other hand, it was much impaired in a patient with untreated subacute bacterial endocarditis of long duration. It was impaired in certain cases of steatorrhoea.

The value, if any, of this test as an aid to the diagnosis of the presence of advanced malignant disease is being investigated.

When citrovorum factor was given *by mouth* to normal persons or to a sprue patient with free hydrochloric acid in the stomach, it appeared in the serum and was excreted in the urine largely as pteroylglutamic acid, as estimated microbiologically. In pernicious anaemia and in a patient without pernicious anaemia but with histamine-fast achlorhydria, it appeared in the serum and was excreted largely as citrovorum factor. In normal persons and in pernicious anaemia patients, citrovorum factor given *by injection* appeared in the serum and urine chiefly as citrovorum factor.

When citrovorum factor was incubated for 2 hours with normal gastric juice at pH 2·8 or with pernicious anaemia gastric juice adjusted to that pH, conversion to pteroylglutamic acid was greater than when citrovorum factor was incubated with pernicious anaemia gastric juice at its pH of 7·8.

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Bile Pigment Formation *in vitro* from Catalase and Peroxidase. By J. E. Kench. (*Nuffield Department of Occupational Health, University of Manchester*)

Many haem derivatives undergo coupled oxidation with ascorbic acid *in vitro* with formation of compounds decomposed by acetic acid to produce bile pigments (Lemberg, Legge & Lockwood, 1939; Kench, Gardikas & Wilkinson, 1950). Catalase and peroxidase are two haemoproteins of particular interest in this connexion, since they are able to decompose H_2O_2, the active agent in the ascorbic acid reaction. Horse-liver catalase itself contains a bile pigment haemoprotein in varying proportion (Lemberg, Norrie & Legge, 1939; Sumner, Dounce & Frampton, 1940), whilst Lemberg & Legge (1943) have demonstrated that ascorbic acid causes inhibition of catalase by accelerating the destruction of

the enzyme by H_2O_2, with production of bile pigment haemoprotein. The interaction of hydrogen peroxide with peroxidase and catalase with formation of complexes has been investigated by Chance (1950) and Keilin & Hartree (1951).

In the present study, horse-liver catalase and horse-radish peroxidase were incubated in air with ascorbic acid at 37° for 2 hr., and resulting bile pigments extracted and examined, as in previous investigations (Kench *et al.* 1950). Horse-liver catalase, Kat. f. 17 500, haematin content 0·76 %, 0·65 mg. haemin/ml. solution, was prepared by the method of Keilin & Hartree (1945). Biliverdin only was obtained by the incubation with ascorbic acid, in yields of 22, 28, 27 (incubated control) and 39 % of original haemin, the latter augmented bile pigment formation arising by addition of 300 mg. ascorbic acid/7 mg. catalase haemin—during 2 hr. incubation.

Horse-radish peroxidase prepared from 20 g. root by the method of Keilin & Hartree (1951), was obtained in a solution, purpurogallin number 225, total enzyme units 517, and containing 0·26 mg./ml. peroxidase haemin. Ascorbic acid treatment gave a yield of biliverdin 2·9 % theoretical, no bile pigment being detected in the control solution.

Spectroscopic observations to detect intermediates in the breakdown of haemin and of methaemalbumin have located increased spectral absorption in the red, 650–700 mμ., not strictly proportional to the yield of bile pigment. Addition of a total of 1 g. ascorbic acid during 2 hr. incubation to unbuffered ferrihaem (14 mg.), either free or combined in methaemalbumin, caused a final pH 4·54 (original pH 7·72), when it was found that 16 and 45 % respectively of the bile pigment yield had been liberated from bile pigment haem, and could be extracted immediately with chloroform.

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The Activity of Acetyl- and Butyrylcholinesterases in some Mammalian Tissues. By A. Todrick.* (*Chemical Defence Experimental Establishment, Porton, nr Salisbury*)

Ord & Thompson (1950) have shown that butyrylcholinesterase activity appears to be relatively high in those tissues where acetyl choline exerts a muscarinic effect. A possible physiological significance has been claimed for this enzyme in one of these tissues (intestine) (Koelle, Koelle & Friedenwald, 1950; Burn, Kordik & Mole, 1952).

In studying the distribution of the two classes of enzyme, certain specific inhibitors have been found valuable. Taking rat brain and intestinal mucosa homogenates as representing the acetyl- and butyrylcholinesterases respectively, it is found that diethazine hydrochloride, 'Diparcol' (cf. Gordon, 1948) and panparnit, 'Parpanit', specifically inhibit the butyrylcholinesterase, while R.P. 3381 and R.P. 3565 (Bovet, Courvoisier, Ducrot & Horclois, 1949) specifically inhibit the acetycholinesterase. The ratio $(I)_{50}$ Brain/$(I)_{50}$ Mucosa is 760 for diethazine hydrochloride, 490 for panparnit, 0·0019 for R.P. 3381 and 0·0057 for R.P. 3565. The usual specific substrates have also been employed.

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Rat heart contains a butyrylcholinesterase which is probably identical with the enzyme of the rat mucosa; dog heart cholinesterase is similar but not identical with these two; the enzyme of goat heart, like that of the serum of ruminants generally, is an acetylcholinesterase.

The cholinesterase distribution has been studied in some nervous tissues of the horse. The sympathetic ganglion, the thoracic sympathetic chain and the trigeminal ganglion contain the acetylcholinesterase almost exclusively, while the ciliary ganglion and nerve may contain both enzymes. The absolute activities of the two latter tissues are low, being only one-twentieth of that of the sympathetic ganglion. This distribution of the two enzymes differs from that found by Koelle (1950) in the rat.

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The Solubilization of Collagen. By D. S. Jackson. (*Rheumatism Research Centre, The University of Manchester*)

It has been shown (Nageotte, 1927) that the collagen of rat-tail tendon can be solubilized by dilute acetic acid and that only a small percentage goes into solution (von Buzagh, 1942).

In an attempt to increase the percentage of collagen dissolved, the effect of bull-testis hyaluronidase was studied. This enzyme is active against chondroitin sulphuric acid, a known constituent of tendon (Meyer, 1945).

Calf tendo Achillis, freed of connective tissue and fat, was incubated for 72 hr. with bull-testis hyaluronidase in acetate buffer (pH 5·2) containing 0·15M-NaCl (*A*). A control batch of tendon was incubated in the same solution with the enzyme omitted (*B*). *A* appeared swollen and the fibres frayed and split up. The tendon was washed with saline, distilled water and acetone, and dried *in vacuo*. 1 g. each of *A* and *B* was shaken with 100 ml. 0·4% (v/v) acetic acid for 24 hr. This was repeated six times. During the first extraction *A* adsorbed considerably more water than *B*. The residues were washed in distilled water and acetone and dried *in vacuo* and weighed. 85% of *A* was solubilized as compared with 25% of *B*.

Collagen fibres were reconstituted from a solution of collagen in 0·4% (v/v) acetic acid, prepared from hyaluronidase-treated tendon:

(*a*) by adding an equal volume of 10% NaCl.

(*b*) by adding a 0·1% solution of potassium chondroitin sulphate, prepared by the method of Einbinder & Schubert (1950) from bovine trachea.

The precipitates were washed in saline, water and acetone, dried *in vacuo*, weighed and shaken with 0·4% (v/v) acetic acid for 24 hr. 95% of the salt-precipitated fibres, and only 3% of the chondroitin sulphate-precipitated fibres were redissolved. After a sample of the latter had been incubated with hyaluronidase virtually all the precipitate was solubilized by 0·4% (v/v) acetic acid.

The results suggest that chondroitin sulphuric acid is responsible for the relative resistance of collagen to solubilization in dilute acetic acid, and may account for the anomalous swelling behaviour at alkaline pH.

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A Quantitative Study in Relation to Electrophoresis of the Staining of Serum Proteins on Filter Paper with Naphthalene Black. By A. L. Latner. (*Section of Chemical Pathology, Department of Pathology, Royal Victoria Infirmary, Newcastle upon Tyne*)

Six rectangular sections of filter paper (Whatman 100, fat-free) were each saturated with a different dilution of the same serum. A seventh section was saturated with 0·9% saline. The papers were dried in a current of air and then in a hot air oven. They were stained by the naphthalene black in wood naphtha (Boots) technique already demonstrated (Latner, 1952).

The papers were rendered translucent with methyl salicylate and their extinction values determined with the Spekker absorptiometer device demonstrated at this meeting, using the transmission of the strip saturated with saline as the 100% transmission value.

On graphing serum-protein concentrations against extinction values, a satisfactory straight line relationship was obtained. Similar results, but not over so wide a range, were obtained with the reflection scanning device previously demonstrated (Latner, Molyneux & Rose, 1952).

The procedure was now repeated using solutions of serum albumin and serum γ-globulin, each con-

taining 0·95 g./100 ml. Almost equal extinction values were obtained indicating within the experimental error of the technique that albumin and γ-globulin have an equal affinity for the stain.

The range of serum dilutions adequately covered the extinction values obtained in the author's technique of quantitative electrophoresis of serum proteins on filter paper (Latner, 1952). It must be emphasized that when using this technique the papers must be rendered absolutely bubble free before scanning by immersion in methyl salicylate in a vacuum desiccator. Also, for quantitative purposes, narrow strips approximating to 2 cm. in width are more satisfactory than those commonly used, which are 5 cm. wide. In the latter there is a tendency to bowing of the protein bands.

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Smooth Muscle Stimulants from the Gastro-Intestinal Tract. Identification of 5-Hydroxytryptamine and its Distinction from Substance P. By C. E. Dalgliesh, C. C. Toh and T. S. Work. (*National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7*)

Extracts of the gastro-intestinal tract contain a number of smooth muscle-stimulating substances including choline, acetylcholine and histamine. In addition to these, several unidentified active substances have been reported. Ivy & Oldberg (1928) attributed the activity to 'cholecystokinin'; Euler & Gaddum (1931) obtained an active extract named 'substance P'; Kokas & Ludany (1933) attributed the activity of their preparation to 'villikinin', and Erspamer (1940) obtained another active extract named 'enteramine'. The evidence that each of these represents a separate substance has not been entirely convincing.

We have been able to show that the enteramine activity of acetone extracts of gastro-intestinal tract is due mainly to 5-hydroxytryptamine but that a second smooth muscle-stimulating substance ('substance C'), probably an indole derivative of related structure, is also present in crude enteramine extracts. By comparing the pharmacological activity of crude 'substance P', partially purified 'substance C', and 5-hydroxytryptamine, we have been able to show that the main activity of 'substance P' is not due to either of the other substances.

Acetone extracts of pig intestine were fractionated by electrodialysis and countercurrent distribution between butanol and water and the various fractions were tested on an isolated atropinized rat colon preparation suspended in the Ca^{++}-deficient salt solution suggested by Gaddum, Peart & Vogt (1949). Two peaks of activity were observed in the countercurrent distribution and material from each of these was further purified by paper chromatography using butanol-acetic acid as solvent. The activity peak partitioning in favour of water contained a substance indistinguishable either by chromatographic methods, colour reactions or pharmacological activity from a synthetic sample of 5-hydroxytryptamine. The activity peak partitioning in favour of butanol, fractionated by paper chromatography, was found to contain traces of 5-hydroxytryptamine (R_F 0·4 in butanol-acetic acid, Whatman paper no. 4), but the main active component had a higher R_F (0·7). It gave colour reactions with the Ehrlich reagent, the Folin-Denis reagent and the Pauly reagent similar to those obtained with 5-hydroxytryptamine.

This investigation confirms the suggestion of Erspamer & Asero (1952) that the substance isolated by them from extracts of the posterior salivary gland of the octopus and from the skin of amphibia and identified as 5-hydroxytryptamine is also the substance responsible for the smooth muscle stimulating properties of acetone extracts of the intestinal tract.

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Adenylpyrophosphates in the Fatty Livers of Guinea-pigs produced by Carbon Tetrachloride. By S. L. ROWLES.* (*Department of Biochemistry, University of Oxford*)

Ennor & Stocken (1948) observed an increased concentration of adenylpyrophosphates (APP) in the livers of guinea pigs treated with carbon tetrachloride, when compared with the livers of 24 hr. starved control animals. This was interpreted as the result of an increased rate of fat oxidation with consequent generation of high-energy phosphate (~ph). Since the increased level of ~ph might be the result of the pathological condition (cf. LePage, 1949) an attempt was made to compare the turnover of ~ph occurring in this tissue with that in normal liver.

Three series of animals, (*a*) treated with carbon tetrachloride by subcutaneous injection, (*b*) 48 hr. starved, and (*c*) normal fed, were injected intravenously with radioactive orthophosphate 15 min. before sacrifice, the animals killed by decapitation, and the livers extracted with trichloroacetic acid (TCA). The APP fraction was separated by precipitation first with barium chloride at pH 8·2 and secondly with mercuric acetate at pH 3·8. The fraction was analysed by enzymic methods (Rowles & Stocken, 1950).

The mean levels of APP labile P in the livers of the three series extracted with ice-cold TCA immediately after excision were 4·5, 2·9, and 3·3 mg./g. liver nitrogen respectively, confirming the increased level in the fatty livers found by Ennor & Stocken. When the liver was frozen in liquid air prior to extraction the APP levels tended to be lower. The livers of all series contained predominantly ATP, the ratio of ATP : ADP being approximately 9 : 1 in terms of labile P.

Data on the incorporation of radioactive P into the phosphate fractions of the livers will be presented. The specific activities of the two labile P groups of APP were generally similar indicating that after a 15 min. period renewal had occurred to about the same extent. In order to compare the turnover of APP labile P in the series the relative specific activities (RSA) with respect to the intracellular orthophosphate were calculated, using chloride space data to correct for the extracellular fraction. The results suggested, however, that the APP labile P was not derived wholly from the intracellular orthophosphate, and the figures are not, therefore, a valid measure of turnover.

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Polymerization of Cottonseed Oil as a Co-factor in Gastric Cancer. By J. G. CHALMERS. (*Cancer Research Department, Royal Beatson Memorial Hospital, Glasgow*)

Tumours of the forestomach have been induced in mice fed on a diet containing heated cottonseed oil. The carcinogenicity of cottonseed oil heated to 320° for 2·5 hr. in the presence of iron and to 350° for 4 hr. without iron has been tested in this way (Peacock, Beck & Chalmers, 1952).

Cottonseed oil polymerizes on heating to 300° in an atmosphere of nitrogen and after 20–25 hr. heating the chief product is an acetone-insoluble polymer. On heating at 350° for 4 hr. there is a marked production of polymeric material which is acetone-soluble and acidic; there is also present a significant amount of ketonic products. On heating at 320° for 2·5 hr. in the presence of iron filings there is a marked increase in the ketonic fraction although polymeric material is still present.

It has been shown that cottonseed oil is decarboxylated on heating with or without a catalyst. Heated alone, the formation of CO_2 has been detected at 345°, a temperature approaching the boiling point of the oil, while in the presence of iron filings CO_2 may be detected at 250°, demonstrating the marked catalytic effect of iron on this reaction.

For comparative purposes examination of a random sample of cottonseed oil used commercially in frying potato crisps is being made. In the factory the oil was heated to 230–250° in an open iron pan. The heated oil was completely soluble in acetone, indicating the absence of acetone-insoluble polymers and a further examination of the acetone-soluble material is being made.

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The Influence of Energy Intake on Ribonucleic Acid Metabolism in Different Fractions of the Liver Cell. By T. W. Wikramanayake, F. C. Heagy and H. N. Munro. (*From the Department of Biochemistry, The University, Glasgow*)

It has been previously reported (Munro, Naismith & Wikramanayake, 1952) that the plane of energy intake influences the rate of phosphorus incorporation into the ribonucleic acid (RNA) of the liver of the rat. The present communication deals with the effect of energy intake on rate of ^{32}P incorporation in different parts of the liver cell. A nuclear fraction, mitochondria, microsomes and a residual cytoplasmic fraction were separated in sucrose from the livers, and in addition nuclei were isolated by the citric acid procedure; in each of these fractions the specific activity of the P of the individual ribonucleotides was determined by the ionophoresis technique of Smellie & Davidson (1952).

Comparisons of ^{32}P uptake were made between rats on low and high energy intakes of a protein-free diet. At 2 hr. after ^{32}P injection the level of energy intake had most influence on P uptake by the RNA of the nuclear fraction. At 4 hr. and at 18 hr. following injection of labelled P, changes in energy intake affected uptake by the RNA of the nuclei, mitochondria and microsomes to a similar extent, but uptake by the RNA of the residual cytoplasmic fraction was much less influenced by the plane of energy intake. This distinctive pattern was not evident in the rate of ^{32}P incorporation into the phospholipins of the same cell fractions.

Quantitative studies of the amount of RNA in these cell fractions at different energy levels have also been made and will be discussed in relation to the data obtained with labelled P.

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DEMONSTRATIONS

The Deoxyribonucleic Acid Content of the Cell Nucleus in Rat Tissues. By S. C. Frazer, R. Y. Thomson and F. C. Heagy. (*Department of Biochemistry, The University of Glasgow*)

The Composition of the Ribonucleic Acids of Liver Cell Nuclei and Cytoplasmic Fractions. By G. W. Crosbie and R. M. S. Smellie. (*Department of Biochemistry, The University of Glasgow*)

The Incorporation of ^{35}S and ^{14}C into the Proteins of Liver Cell Nuclei and Cytoplasmic Fractions. By G. Leaf, W. M. McIndoe and R. M. S. Smellie. (*Department of Biochemistry, The University of Glasgow*)

Cortisone, Growth Hormone, and Insulin Action of Cultures on Chick Embryo Heart. By I. Leslie. (*Department of Biochemistry, University of Glasgow*)

Conversion of the Spekker Absorptiometer to a Semi-automatic Scanning Device for Quantitative Electrophoresis of Serum Proteins on Filter Paper. By A. L. Latner. (*Section of Chemical Pathology, Department of Pathology, Royal Victoria Infirmary, Newcastle upon Tyne*)

The absorptiometer has been mounted on a rectangular wooden base and modified by replacing the selenium barrier-layer cells with photo valves. An amplifying bridge circuit (Baldwin photometer) is used for balancing.

For the scanning of stained paper electrophoresis strips only one half of the instrument is used and full scale deflection on the milliammetre adjusted by a sensitivity device to correspond to 100% transmission.

A rectangular vessel, 24 cm. long, 1·2 cm. wide and 2·5 cm. deep (internal), has been mounted on a brass plate which slides into the grooves normally occupied by the device for carrying the absorptiometer cells. The vessel has two glass walls to allow light to pass through it. It is partially filled with methyl salicylate. The electrophoresis strip, previously stained with Naphthalene Black 12 B 200, then immersed in methyl salicylate and rendered bubble free (Latner, 1952) is sandwiched between the distal glass wall and a rectangular glass plate held vertically in position by two phosphor-bronze springs. The vessel is now filled with sufficient methyl salicylate to reach the level of the upper edge of the paper strip.

A removable slit, 1 cm. long and 2 mm. wide, and an Ilford 607 filter are mounted between the lamphouse and the vessel carrying the paper. The latter can therefore be scanned by pushing the brass mount along the grooves. The mount has attached at a right angle to one end a ruler which passes over a sheet of graph paper fixed on to a platform, which is mounted on the same wooden base as the absorptiometer. The ruler is calibrated in such a way that milliammeter readings are directly converted into extinction values.

The vessel containing the paper strip is advanced past the slit 2 mm. at a time and each reading plotted by hand on to the graph using the ruler. In this way a complete electrophoresis curve is obtained in 5 to 10 min.

The author wishes to express his gratitude to Dr F. T. Farmer and Mr M. J. Day of the Royal Victoria Infirmary for much help in the construction of the apparatus.

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A Preparative Paper-Electrophoresis Technique and Its Application to Vitamin B_{12}-Binding in Serum. By A. L. Latner, Laureen Raine, G. I. M. Ross and C. C. Ungley. (*Royal Victoria Infirmary, Newcastle upon Tyne, and the Postgraduate Medical School of London*)

Whatman 31 (extra thick) filter papers are used. They are 38 cm. long × 5 cm. wide. A pencil line is drawn which divides the paper into two 19 cm. lengths. This is the line of application.

Commencing 1 cm. from this line ten additional parallel lines 1 cm. apart are drawn on the cathode side and a further fifteen lines similarly placed on the anode side. The papers are each immersed in a barbitone buffer at pH 8·6, blotted to remove excess of liquid and placed in the electrophoresis apparatus. They are left for 1 hr. to equilibrate.

Serum, previously concentrated to a small volume by ultra-filtration, is applied with a paint brush to the line of application. In this way, using six papers and applying 0·5 ml. to each, it is possible to apply serum which had an initial volume of 10 ml. Electrophoresis is carried out for 16 hr. at 120 V. using an ordinary high-tension battery.

Each paper successively is now removed and cut into the twenty-five segments previously defined. Each segment is rapidly placed into one of twenty-five screw-capped bottles. Corresponding segments from each paper are placed in corresponding bottles. To remove the liquid from the paper, the segments in each bottle are rapidly removed and placed in a clean dry watch glass. The mouth of the bottle is covered with a piece of nylon tricot, the paper segments placed on top of this and the whole held in position by screwing on the cap. The bottles are now centrifuged at 3000 rev./min. for 10 min. Each bottle now contains approximately 1·5 ml. of liquid. The fractions carrying bile pigments and the brown iron-containing pigment can easily be seen.

Using *Euglena gracilis*, the material in each bottle can be assayed for vitamin B_{12}. These methods have demonstrated the position of the protein fractions to which vitamin B_{12} is bound in serum *in vivo* or *in vitro*. Normal and pernicious anaemia sera have been compared.

The Detection and Identification of *iso*Nicotinic Acid Hydrazide and Other Tertiary Pyridine Derivatives. By W. F. J. CUTHBERTSON and D. M. IRELAND. *Glaxo Laboratories Ltd., Greenford, Middlesex*)

Chromatography. Huebner (1951), Kodicek & Reddi (1951) and Wollish, Schmall & Shafer (1951) described the chromatographic separation of tertiary pyridine derivatives on paper and their identification by the König (1904) reaction. We found water-saturated *n*-butanol and *n*-propanol/water (4:1, v/v) were the best of the solvent systems tested by downward development (Whatman no. 1), though some tailing occurred with the hydrazides. The R_F values (*n*-butanol) were: nicotinic acid (NA) 0·14, *iso*nicotinic acid (INA) 0·14, nicotinamide (N) 0·6, *iso*nicotinamide (IN) 0·6, nicotinic acid hydrazide (NAH) 0·4, *iso*nicotinic acid hydrazide (INAH) 0·43; in *n*-propanol; NA 0·4, INA 0·4, N 0·72, IN 0·72, NAH 0·6, INAH 0·62, *iso*propyl*iso*nicotinic acid hydrazide (PINAH) 0·91 and hydrazine 0·0.

Colour development. Picryl chloride, used in a hydrazine test (Kuborg & Cherkesov, 1951, 1952) gave coloured products with tertiary pyridine compounds, but only those derived from the hydrazides remained at acid pH. Chromatograms were rapidly dipped in ethanolic picryl chloride (1·5% w/v) and allowed to dry. Exposure to ammonia vapour caused red spots to develop when 5μg. pyridine, NA, INA, N or IN were present, but only the red-purple derivatives of INAH, NAH and PINAH remained after exposure to acetic acid vapour; in this way the hydrazides are readily distinguished.

After treatment of paper chromatograms with CNBr (Wang & Kodicek, 1943) INAH, NAH and PNAH (5μg.) fluoresced blue in ultraviolet light (254 mμ.), thereby differing from all other tertiary pyridine derivatives tested. Trial of a series of aromatic amines and 1-phenyl-3-methyl pyrazolone (PMP) (Knorr, 1883; Epstein, 1947) in the König (1904) reaction showed that NA, N and NAH were best detected by their yellow derivatives (which fluoresced yellow in ultraviolet) formed on spraying with the *p*-aminobenzoic acid reagent of Kodicek & Reddi (1951) after treatment with CNBr. Spraying with benzidine (Huebner, 1951) after CNBr treatment yielded a purple derivative with 3–5μg. IN and a blue colour with 10μg. INA. All the nicotinyl and *iso*nicotinyl series (5μg.) yielded distinctive colours on spraying with PMP (sat. aq. soln.) after CNBr: NA orange, N red, NAH yellow, INA purple; IN purple, INAH green with blue fluorescence in ultraviolet and PINAH yellow-green with blue fluorescence in ultraviolet.

INAH metabolites. Two spots corresponding in R_F and colour reactions to INA and INAH appear on chromatographing butanol extracts of urines obtained from cats after injection (i.p.) of INAH. Other compounds detectable after treatment with picryl chloride, or CNBr followed by PMP, have also been observed in human and cat urines after doses of INAH.

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Nicotinic acid	5·0	10·0	25·0
Nicotinamide	5·0	10·0	25·0
Calcium *d*-pantothenate	1·0	5·0	10·0
Pyridoxine hydrochloride	0·5	1·0	5·0
d-Biotin	0·1	0·5	1·0
p-Aminobenzoic acid	1·0	5·0	10·0
Choline chloride	5·0	10·0	25·0
Inositol	5·0	10·0	25·0
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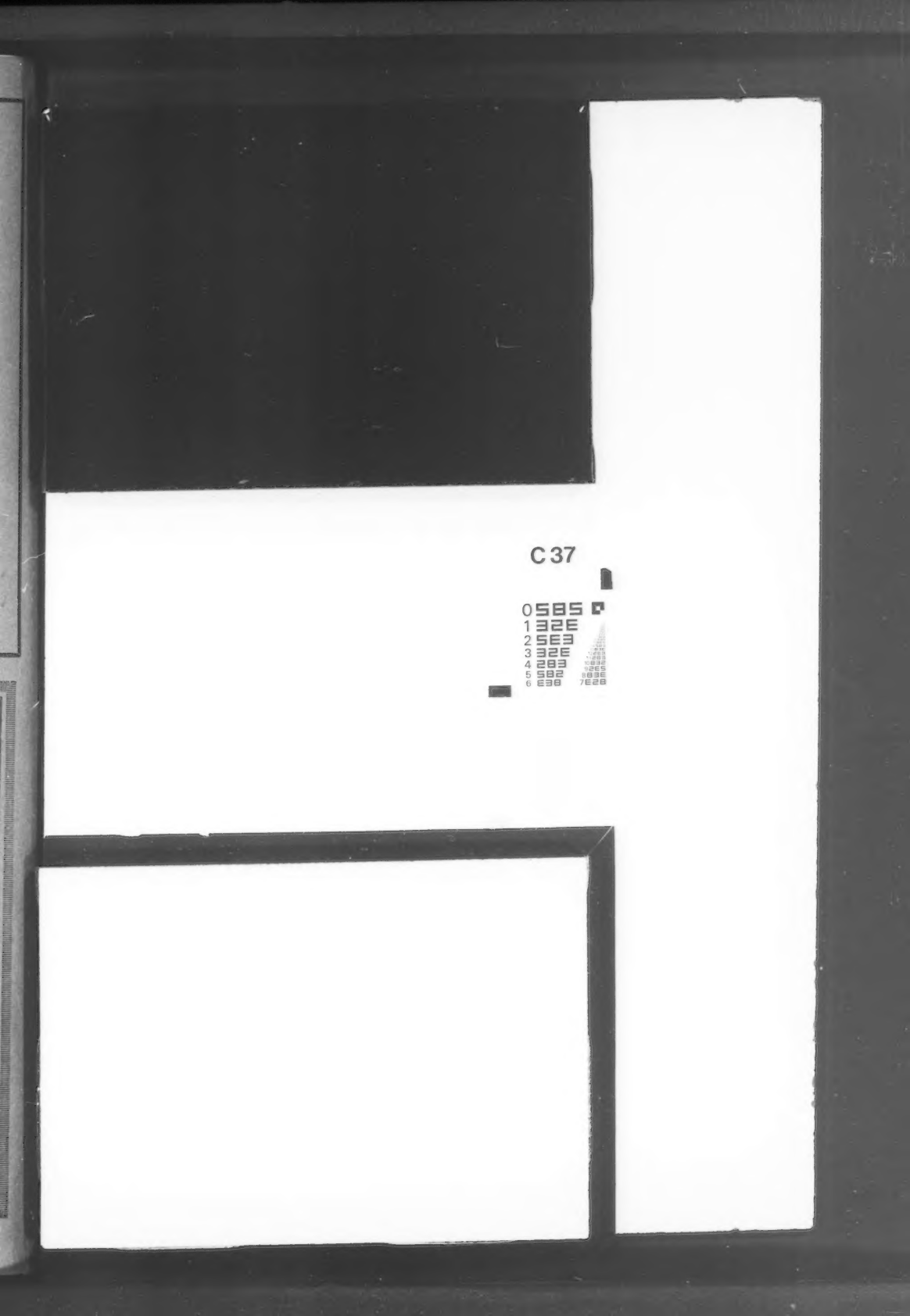
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